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Studies on the hydrolysis of metal ions VII

The hydrolysis of the iron(III) ion, Fe^{3+}

By Bengt O. A. Hedström

With 9 figures in the text

The equilibria in hydrolyzed iron(III) solutions have been the subject of numerous investigations. Most workers in the field have employed electrochemical (*1*, *4*, *6*, *7*, *8*, *10*, *14*, *16*) or spectrophotometric (*14*, *17*, *18*, *20*, *21*) methods and the lack of agreement between the results obtained by different authors is so striking (see table 1), that it suggests that the hydrolysis of Fe^{3+} is a complicated process, and that the experimental data have been inadequate to provide conclusive information about the actual equilibria existing in hydrolyzed iron(III) solutions. However, in recent years new electrochemical methods of studying complex equilibria have been developed (*3*, *11*, *15*), and these are well suited to the study of even complicated hydrolytic reactions. It therefore seemed possible to investigate in more detail the

Table 1

Survey of previous investigations on the hydrolysis of the iron(III) ion, Fe^{3+}.

$\varkappa_{11} = [H^+]\,[FeOH^{2+}]\,/\,[Fe^{3+}]$; $\varkappa_{12} = [H^+]^2\,[Fe(OH)_2^+]\,/\,[Fe^{3+}]$; $\varkappa_{22} = [H^+]^2\,[Fe_2(OH)_2^{4+}]/[Fe^{2+}]^2$

Author	$10^3\varkappa_{11}C$	$10^6\varkappa_{12}C^2$	$10^3\varkappa_{22}C$	Temp. °C	Ionic strength, C	Method	Year
Arden (*1*)	1.25	0.53	–	25	not controlled	emf	1951
Bjerrum, N (*4*).	2.48	–	–		varied from 0.020 to 0.0003	conductivity	1907[1]
Bray & Hershey (*5*) .	6.0 ± 0.5	–	–	25	zero	spectrophot.	1934
Brosset (*6*)	1.73 ± 0.01	–	–	25	0.550	emf	1941
Lamb & Jaques (*14*) ..	2.5	0.05	–	25	zero	emf	1938
Lindstrand (*16*)	11	–	–	25	zero	emf	1939
	18	–	–	(25)	zero	emf	1939[2]
Siddal & Vosburgh (*20*)	6.5 ± 0.4	–	–	25	zero	spectrophot.	1951
This work	0.90 ± 0.10	0.47 ± 0.10	1.22 ± 0.10	25	3.000	emf	1952

[1] Based upon Goodwin's measurements (*10*).

[2] Recalculated from Brönsted & Volquartz measurements at 18 °C (*7*).

hydrolysis of the iron(III) ion, Fe^{3+}. Apart from being of general interest this process is of some practical importance, e.g., in the production of iron oxide pigments and the study of the corrosion of iron and steel.

Method of measurement

The measurements were carried out at 25.0° C, and all solutions were made so as to contain 3000 mC (millimoles/liter) ClO_4^-. This high ionic strength was chosen because $[H^+]$ was to be rather high in some solutions, and because of the presence of relatively highly charged ions (Fe^{3+} and its hydrolysis products). As in the study of the hydrolysis of the iron(II) ion (*12*), it is believed that any complex formation involving ClO_4^- need not be taken into account in the interpretation of the hydrolysis data, and that activity coefficients are constant. This implies that concentrations instead of activities can be used in the equilibrium equations.

The equilibrium solutions studied contained both Fe^{2+} and Fe^{3+}, each kept at a fixed, total concentration in each series of experiments. The hydrolysis of Fe^{2+} is not noticeable at $pH < 5$ (*12*), whereas the iron(III) ion hydrolyzes in quite another, lower, pH range. For this reason, $[Fe^{2+}]$ can be expected to be constant in each series of experiments.

The (free) iron(III) ion concentration was measured with a bright platinum foil electrode; the hydrogen ion concentration with a glass electrode. A calomel electrode of the following composition served as a reference electrode:

$$\mathrm{CE}| = \mathrm{Hg, Hg_2Cl_2} \,|\, 4\ \mathrm{C\ NaCl} \,|\, 3\ \mathrm{C\ NaClO_4}|$$

Measured emf:s refer to the following two types of cells:

$$-\ \mathrm{CE}\,|\,\mathrm{H^+}\,|\,\text{glass electrode} +,$$

and

$$-\ \mathrm{CE}\,|\,\mathrm{Fe^{3+}, Fe^{2+}}\,|\,\mathrm{Pt} + .$$

For the first cell we have:

$$E_g = E_{go} + 59.16 \log h + E_j \tag{1}$$

where E_g is the observed glass electrode potential (in mV), E_{go} a constant, h the hydrogen ion concentration (in mC) and E_j the liquid junction potential.

For the second cell we have:

$$E = E_0 + 59.16 \log ([Fe^{3+}]/[Fe^{2+}]) + E_j. \tag{2}$$

where E is the measured redox emf and E_0 a constant.

Since the total iron(III) and iron(II) concentrations are constant in each series of measurements, Equation 2 can be written:

$$E' = E - E_j = E_0 - 59.16 \log [Fe^{2+}]_{tot}/c + 59.16 \log M/c,$$

where c is the total iron(III) concentration, and $M = [Fe^{3+}]$. This is conveniently written as

$$E' = E_0' + 59.16 \log \beta \tag{3}$$

where $E_0' = E_0 - 59.16 \log [Fe^{2+}]_{tot}/c$, and $\beta = M/c$, is the fraction of the total iron(III) concentration which is not hydrolyzed.

$E_{\mathrm{g}0}$ and E_0' were determined from the first part of each titration, where h was relatively large and therefore the hydrolysis could be neglected, i.e., $\beta \approx 1$. In this range $E' \approx E_0'$ and $h \approx H$, the hydrogen ion concentration added.

Biedermann and Sillén (*2*) have shown that E_{j} is a function of h only, if the concentrations of the metal ions studied are low in comparison with the total ionic strength. The E_{j} values were taken from their paper. In the h range studied in this work, $E_{\mathrm{j}} = -0.018\ h$, as a very good approximation.

The titration technique employed involved the following steps. Equal volumes of 3000 mC $NaClO_4$ and acid iron(III) – iron(II) solution (3000 mC ClO_4^-) were mixed in the titration vessel. Then the hydrogen ion concentration was lowered stepwise by the addition from burettes of equal volumes of base and iron solution. The base used, for reasons discussed under "Measurements", was HCO_3^-.

From the glass electrode and redox potentials h and M were calculated for each equilibrium solution. From analysis the values of H and c were known. The interpretation of these figures is discussed in the next section, and, in more detail, under "Discussion", when the experimental results have been presented.

Method used in the evaluation of equilibrium constants

At an early stage of the experimental work it became apparent that the iron(III) ion hydrolyzes with the formation of polynuclear complexes. It was therefore found necessary to find suitable mathematical devices, capable of analyzing the rather complicated data in an unambiguous way. Some of the results of this theoretical work will form the subject of a separate paper (*13*).

Consider the equilibrium between a positive ion M^{z+}, water, and their reaction products:

$$pM^{z+} + q\,H_2O \rightleftharpoons M_p(OH)_q^{(pz-q)+} + qH^+$$

A series of equilibria of this type may be present, since p and q may, in general, have any integer values. On applying the law of mass action (assuming activity coefficients to be constant) equations of the following type are obtained:

$$h^q\,[M_p(OH)_q^{(pz-q)+}]\,/\,M^p = \varkappa_{pq} \tag{4}$$

where $M = [M^{z+}]$ and $\varkappa_{pq}$ is the equilibrium constant. From the law of conservation of mass we find:

$$c = M + \sum_{(p,\,q)} p\,\varkappa_{pq}\,M^p\,h^{-q} \tag{5}$$

where c denotes the total, stochiometrical metal ion concentration. On rewriting Equation 5 there is obtained:

$$h(c - M)\,/\,M = h(1\,/\,\beta - 1) = h\,b = \sum_{(p,\,q)} p\,\varkappa_{pq}\,M^{p-1}\,h^{1-q} \tag{6}$$

where $b = (1/\beta - 1)$ is the ratio of the fraction hydrolyzed to the fraction not hydrolyzed. If only mononuclear complexes are formed, β, b and $b\,h$ will be functions of h only, i.e., independent of M (and c), since $p = 1$. On the other hand, in the case of a polynuclear process $b\,h$ will be a polynomial function of M only, as long as h is kept at a constant value. The first fact enables us to decide between mono- or polynuclear reaction: If we plot β against h (or some suitable function of h, for instance $\log h$) and get the same curve for different c values, then the reaction mechanism is a mononuclear one; if the β curves differ, then polynuclear complexes must also be present in the solutions.

In order to evaluate $p_{\max}$ (the "degree" of the polynuclear process), we may plot bh against M at constant h. If we get a straight line with a bh intercept and a finite slope, then only mono- and dinuclear complexes are formed; if a curve of the second degree, trinuclear complexes also, and so on. In the case of a pronounced and complicated polynuclear process (high p values) it might in practice be difficult to evaluate $p_{\max}$, and in such cases it might be more practical to try some specific, plausible reaction mechanism, form the most suitable characteristic function, and test the data against it. As in the case of the hydrolysis of Bi^{3+} (*11*), it might even be necessary to choose an "infinite" reaction mechanism ($p_{\max} = \infty$).

Now, for each p value, q may, in general, take on more than one value. In order to find all the equilibrium constants, we must study the differences between the (bh, M) curves obtained at different (constant) values of h. To make this point clear, let us consider a reaction mechanism in which $p_{\max} = 2$.

In general, for a dinuclear process, we shall have to consider the following equlibria.

$$\begin{array}{ll}
 & \text{Equilibrium constant} \\
M^{z+} + H_2O \rightleftharpoons MOH^{(z-1)+} \quad + H^2, & \varkappa_{11} \\
M^{z+} + H_2O \rightleftharpoons M(OH)_2^{(z-2)+} + 2H^+, & \varkappa_{12} \\
\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots & \\
2M^{z+} + H_2O \rightleftharpoons M_2OH^{(2z-1)+} + H^+, & \varkappa_{21} \\
2M^{z+} + 2H_2O \rightleftharpoons M_2(OH)_2^{(2z-2)+} + 2H^+, & \varkappa_{22} \\
\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots &
\end{array}$$

If in Equation 6 $p_{\max}$ equals 2 we obtain:

$$bh = \varkappa_{11} + \varkappa_{12} h^{-1} + \varkappa_{13} h^{-2} + \ldots 2M(\varkappa_{21} + \varkappa_{22} h^{-1} + \varkappa_{23} h^{-2} + \ldots). \tag{7}$$

If bh is plotted against M at a certain constant value of h we get a straight line with intercept $(\varkappa_{11} + \varkappa_{12} h^{-1} + \ldots)$ and slope $2(\varkappa_{21} + \varkappa_{22} h^{-1} + \ldots)$. If a number of $\varkappa$'s must be considered, we shall have to use a number of bh *vs.* M curves for different values of h, in order to be able to calculate all $\varkappa$'s from the resulting system of equations.

The data on the hydrolysis of the iron(III) ion which follow, can be explained by introducing only three constants ($\varkappa_{11}$, $\varkappa_{12}$, and $\varkappa_{22}$). Further discussion on the evaluation of these constants is postponed until the experiments have been described.

Apparatus

The apparatus used in the emf measurements was essentially the same as that used in the work on the hydrolysis of the iron(II) ion (*12*).

Two different glass electrodes were used. One was a Radiometer glass electrode (here denoted R), the other (B) was made by E. Blomgren and his coworkers in Uppsala.

Two bright platinum electrodes were used simultaneously. Before use these electrodes were boiled in concentrated nitric acid, rinsed with distilled water and heated to red brightness in an alcohol flame.

Nitrogen gas was passed through the solutions during the emf measurements. Regarding the purification of the nitrogen, reference is made to the paper on the iron(II) ion (*12*).

Chemicals and analysis

The mixed, acid iron(II) and iron(III) perchlorate stock solution was prepared from perchloric acid (A. R.) and iron(II) and iron(III) perchlorates (manufactured by G. F. Smith Chemical Company). The stock solution gave no precipitate with $BaCl_2$, and (after oxidation of Fe^{2+} with H_2O_2) only an extremely faint bluish colouration with $AgNO_3$.

The ClO_4^- concentration of the stock solution was determined by the ion exchange method. The total iron concentration was determined in the usual way by passing a sample through a cadmium reductor and titrating with $KMnO_4$. The iron(II) concentration was determined by direct titration with $KMnO_4$. $[Fe^{3+}]$ was determined by difference ($[Fe]_{total} - [Fe^{2+}]$), the hydrogen ion concentration also by difference ($[ClO_4^-] - 3[Fe^{3+}] - 2[Fe^{2+}]$).

The iron solutions used in the experiments were made so as to contain exactly 3000 mC ClO_4^-, using $NaClO_4$ as an inert electrolyte.

The $NaHCO_3$ solution ($[ClO_4^-] = 3000$ mC) used as a base in the emf titrations was standardized against $HClO_4$, using methyl red as indicator. Near the equivalence point the acid was added dropwise; after each addition the solution was freed from CO_2 by boiling for a few minutes. When the indicator first changed colour at high temperature, the solution was cooled, and a drop of 100 mC NaOH was added. The faintly red coloured solution turned yellow.

For details on the standardization of the titration solutions, the reader is referred to (*12*).

Measurements

Sodium hydroxide (≈ 200 mC) was first tried as the base in the titrations, but it was unsatisfactory. Because of the local excess of base in the solutions, a gelatinous precipitate was formed at each addition of NaOH solution. In spite of vigorous agitation, the precipitate dissolved only slowly in the relatively weakly acid solutions used. The redox and glass electrodes therefore did not attain constant potentials. Much lower sodium hydroxide concentrations could not be used for practical reasons, since the volumes to be handled would have been too large.

Sodium hydrogen carbonate solution, on the other hand, did not precipitate any iron except, of course, in the most weakly acid solutions when a solid phase would in any case be present at equilibrium. The electrodes attained a steady potential in about ten minutes, on the average, after each addition from the burettes. Sodium hydrogen carbonate was therefore used in all the titration experiments to be described.

The carbon dioxide formed during the titration was expelled by the rather rapid stream of nitrogen gas.

Even if CO_2 or its hydrolysis products have a tendency to form complexes with iron ions, it would not be expected to do so in the solutions studied, since the nitrogen passed through the acid solutions would, in the long run, displace the equilibrium so that the concentration of any possible complexes would be very small. In order to get an estimate of the extent of any possible complex formation, the following experiment was carried out.

At the end of one titration experiment (titration No. 11, table 2) the titration apparatus was left overnight with nitrogen gas bubbling through the solution. The next morning the potential of the Pt electrodes was exactly the same as the night before, showing that equilibrium had been attained. Now a rapid stream of carbon dioxide, taken from a cylinder, was passed through the solution. The redox potential increased by 0.4 mV. When the carbon dioxide stream was stopped and nitrogen gas again passed through the solution the potential immediately became the same as before the carbon dioxide treatment. The first slight increase in the redox potential may be due to the formation of a weak complex between CO_2, or its hydrolysis products, and Fe^{2+}. However, this effect can be safely disregarded under the conditions prevailing in the measurements.

Differences between the potentials of the two platinum electrodes used simultaneously could never be observed. One glass electrode was used at a time. It was tested against a quinhydrone electrode by a separate acid-base titration. Both glass electrodes seemed to have a "slope" close to the theoretical value, 59.16 mV, i.e., their potentials ran parallel to that of the quinhydrone electrode, within the experimental error. Although the two electrodes did not give exactly the same results when used in the iron solutions under identical conditions, the deviations were small, and do not invalidate the conclusions drawn from the measurements.

The glass electrodes seemed to reach their equilibrium potentials in about the same time as the redox electrodes.

At the start of a titration experiment 25.00 ml of 3000 mC $NaClO_4$ was mixed with 25.00 ml of iron solution in the titration vessel. (In some cases (e.g. table 3) the sodium perchlorate was added in portions; in this way a few more points were obtained from which E_{0g} and E_0' could be determined). Then equal volumes (v_B ml) of base and iron solution were added in portions from the burettes. The hydrogen ion concentration added, H mC, was calculated from

$$H = \frac{25 H_M - v_B(B - H_M)}{50 + 2\,v_B}, \tag{8}$$

where H_M is the analytically determined hydrogen ion concentration of the acid iron solution used, and B(mC) the concentration of the base solution.

For each point of the titrations the following quantities were calculated: H, h, β, $\log h$, hb, M, and $Z = (h - H)/c$. The quantity Z is clearly equal to the average number of hydrogen ions set free per iron(III) ion.

The titrations were continued until the potentials did not attain constant values, indicating that a precipitate or a colloidal suspension had formed. No attempts were made to estimate the solubility product, because the author feels that this might be so difficult a task that it should form the subject of a separate investigation.

Table 2

Survey of the titrations. (Concentrations in millimoles/liter)

No	Symbol	$2c$ mC	$2[Fe^{2+}]$ mC	H_M mC	B mC	v_B max, ml	Glass electrode	E_{go} mV	E_0' mV	E_0 mV
1	○	0.200	0.1079	76.9	195.1	32	B	− 40.8	541.6	525.7
2	●	0.200	0.0236	77.6	135.1	32	B	− 39.3	579.9	535.0
3	◓	0.400	0.0471	80.4	135.1	35	B	− 40.2	593.2	538.3
4	◒	0.400	0.2158	78.3	135.1	35	B	− 40.3	546.6	530.7
5	+	2.00	1.079	123.4	252.2	23	B	− 36.4	533.9	518.0
6	×	4.00	2.160	140.0	256.6	28	R	− 37.1	535.6	519.7
7	■	10.00	1.178	150.0	270.2	32	B	− 40.5	574.1	519.2
8	□	10.00	5.40	150.0	256.6	36	R	− 33.2	533.5	517.6
9	◇	20.00	10.79	150.0	256.6	38	R	− 35.7	532.2	516.3
10	◆	50.00	26.98	149.0	256.6	50	R	− 34.5	530.5	514.6
11	▲	100.0	11.30	129.3	252.2	90	B	− 34.0	573.6	518.7
12	△	100.0	53.96	145.9	246.2	65	R	− 36.0	528.5	512.6

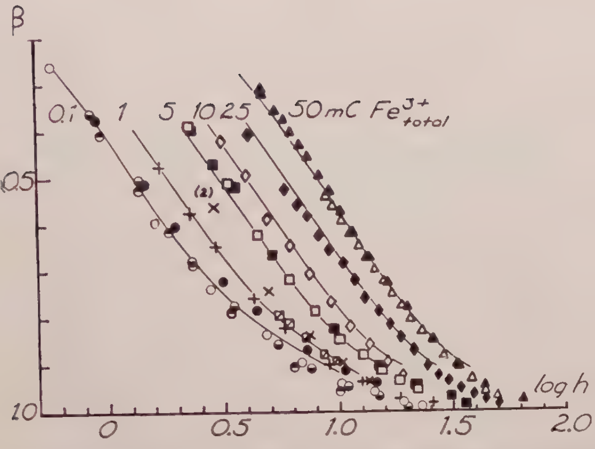


Fig. 1. β *vs.* log h for different, total iron(III) concentrations. The curves have been calculated from the following equilibrium constants: $\varkappa_{11} = 0.90$ mC, $\varkappa_{12} = 0.49$ (mC)^2, $\varkappa_{22} = 1.22$ mC.

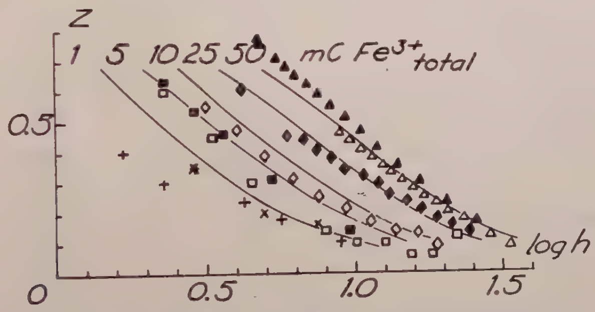


Fig. 2. Z *vs.* log h for different, total iron(III) concentrations. The curves have been calculated from the same equilibrium constants as those used in Figure 1.

Table 2 contains a survey of the titrations. In order to save space, only the titrations No. 1 and 12 are given (tables 4 and 3). These titrations can be regarded as representative. The complete data can, however, be presented graphically in a very condensed form. This has been done in Figures 1 and 2, where β and Z have been plotted against log h. These Figures form the basis of the following discussion on the reaction mechanism and the evaluation of the equilibrium constants.

Discussion

In a recent paper on the hydrolysis of iron(III) sulphate solutions, Arden (*1*) expresses the opinion that "the complete hydrolysis of ferric sulphate is a slow process, and pH measurements vary according to the moment of determination. A study of the reactions of ferric sulphate must therefore include the effect of time, and a normal titration technique, even if carried out at a known rate, must lead to false conclusions, since the observations are made progressively later as the titration proceeds, and the solutions are in correspondingly different equilibrium states".

Table 3

Titration No. 12 (For further details, see Table 2).

a) 25.00 ml iron solution + v ml 3000 mC $NaClO_4$. $(H = 145.9 \cdot \frac{25}{25+v}$ mC).

v ml	Eg, mV	Ej, mV	H, mC	E, mV
0	+87.4	2.3	145.9	
5.00	83.4	2.0	121.6	+526.4
10.00	80.5	1.7	104.2	527.0
15.00	77.5	1.5	91.19	527.1
20.00	75.0	1.3	81.06	527.1
25.00	73.0	1.2	72.95	527.4

b) $+v_B$ ml base $+ v_B$ ml iron solution $(H = \frac{25 \cdot 145.9 - v_B\ (B - 145.9)}{50 + 2v_B}$ mC).

v_B ml	Eg, mV	Ej, mV	H, mC	E, mV	h mC	β	Z
2.00	70.2	1.1	63.8	527.4	65.1	1.000	0.026
4.00	67.5	0.9	56.0	527.2	58.4	0.985	0.016
7.00	63.5	0.8	46.0	526.9	49.8	0.969	0.032
10.00	60.3	0.7	37.8	526.7	43.8	0.956	0.042
13.00	57.0	0.6	36.48	526.1	38.4	0.932	0.068
16.00	53.6	0.5	24.91	525.4	33.5	0.904	0.096
19.00	50.0	0.5	19.80	524.8	29.0	0.883	0.118
22.00	47.0	0.4	15.33	523.9	25.7	0.849	0.152
25.00	44.5	0.3	11.40	523.2	23.3	0.826	0.174
28.00	41.5	0.3	7.925	522.5	20.7	0.801	0.200
31.00	39.1	0.3	4.813	521.6	18.8	0.773	0.228
34.00	31.8	0.3	2.017	510.8	17.2	0.750	0.250
37.00	34.5	0.3	−0.508	519.8	15.7	0.721	0.278
40.00	32.2	0.3	−2.800	518.9	14.4	0.697	0.304
43.00	30.2	0.2	−4.893	518.0	13.2	0.670	0.330
46.00	28.5	0.2	−6.805	517.1	12.4	0.647	0.354
50.00	26.4	0.2	−9.113	516.0	11.4	0.619	0.382
55.00	24.0	0.2	−11.68	514.8	10.4	0.591	0.410
60.00	21.8	0.2	−13.94	513.4	9.55	0.560	0.440
65.00	20.0	0.2	−15.96	512.4	9.91	0.538	0.462

This is undoubtedly so, if one uses a *strong* base in the titrations. However, by using HCO_3^- this difficulty may be easily overcome, at least in perchlorate solutions (see under "Measurements").

One more experimental fact may be cited in favour of this contention. In Figure 1 the points with symbols ⧄ correspond to a titration at $c = 2.2$ mC. Unlike all the other titrations, this titration was made in a reversed order by adding acid to the partly hydrolyzed iron solution prepared by diluting the stock solution. Since the points corresponding to this titration and those corresponding to $c = 2.0$ mC do not differ significantly, we may conclude that the measured values correspond to true equilibria.

On the basis of a spectrophotometric study on mixtures of iron(II) and iron(III) in hydrochloric acid solutions, McConnell and Davidson (*17*) claim that "interaction complexes" containing both iron(II) and iron(III) are formed. "This theory leads

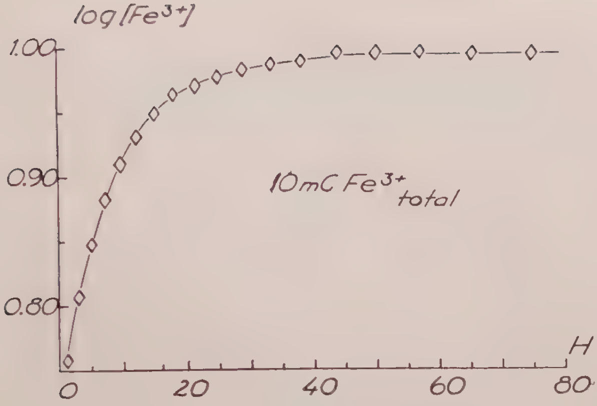


Fig. 3. log M *vs.* H for $c = 10$ mC.

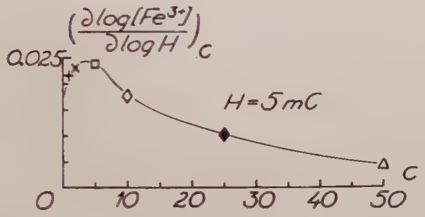


Fig. 4. $(\partial \log M / \partial \log H)_c$ *vs.* c for $H = 5$ mC.

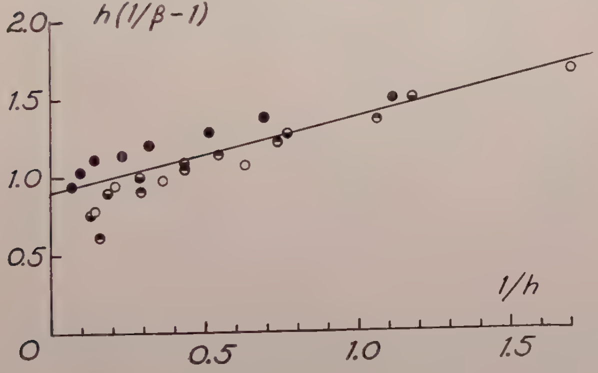


Fig. 5. $h(1/\beta - 1) = h\,b$ *vs.* $1/h$ for the c values 0.1 mC (○, ●) and 0.2 mC (◓, ⊖).

to the suggestion that interaction absorption due to hydroxyl bridging may occur in mixed solutions of $Fe(ClO_4)_2$ and $Fe(ClO_4)_3$ which are less acid than the ones studied here."

However, the titrations at $c = 5$ mC and $c = 50$ mC, Figure 1, provide no support for the assumption that "mixed" complexes, containing both Fe^{2+} and Fe^{3+}, were formed in the solutions studied in this investigation. In spite of entirely different iron(II) concentrations the agreement between titrations at the same c values is

Table 4

Titration No. 1 (For further details see Table 2).

v_B ml	Eg mV	Ed mV	H mC	E mV	β	bh
0.00	+52.4	0.6	38.45	+541.1		
2.00	48.9	0.5	33.45	541.0		
4.00	45.2	0.5	29.13	541.0		
7.00	40.1	0.4	23.67	540.9	0.988	0.284
10.00	34.7	0.4	19.15	540.7	0.981	0.364
14.00	27.0	0.3	14.20	540.2	0.958	0.625
18.00	18.6	0.2	10.17	539.8	0.940	0.640
22.00	8.5	0.1	6.83	538.7	0.897	0.785
25.00	−0.6	0.1	4.675	536.9	0.836	0.941
28.00	−14.7	0.1	2.763	533.7	0.738	0.983
30.00	−29.0	0.1	1.605	528.2	0.596	1.078
32.00	−54.5	0.0	0.527	507.0	0.260	1.673

very good. If β is plotted against H, the agreement is almost perfect, showing that the spread in the β (log h) plots is primarily due to differences in the glass electrodes used.

In titrations 1 to 4 a more accurate H_M value was calculated from the measured glass electrode potentials. This correction did not, however, noticeably change the h values, as determined from the glass electrodes, and therefore did not affect the following evaluation of the equilibrium constants.

As can be seen from Table 2, E_0 is subject to some variation. There are probably two reasons for this. One is the "secular" fluctuations in the potential of the calomel electrode. Another reason may be the slow oxidation of Fe^{2+} during storage of the iron solutions, which would cause E_0, as calculated, to be too high. Whilst in the titrations at relatively high c values (No. 5 to 12) the E_0 variation may be very well explained by the first factor, oxidation of Fe^{2+} during storage seems to be the predominating factor in the titrations No. 1 to 4, where $[Fe^{2+}]$ is very low. However, even if in these solutions 50 % or more of the iron(II) ions has been oxidized during storage, the measured (β, h) values would not be noticeably affected. This can be best seen from Figure 1, which shows that the data for $c = 0.1$ and $c = 0.2$ mC do not differ significantly. The most important thing is, of course, that, due to the presence of an inert atmosphere, no Fe^{2+} has been oxidized during the actual titrations. The consumption of hydrogen ion due to the oxidation of Fe^{2+} during storage was considered by correcting H_M as described above.

Since the greatest source of error seemed to be the glass electrodes, it was desirable to have an independent check of at least some of the measured h values. Use was therefore made of the following equation, derived in another paper (*13*):

$$\log h = \left[\int_0^c (\partial \log M / \partial H)_c \, \mathrm{d}c \right]_H + \log H \tag{9}$$

Plots of log M against H were prepared for the c values 1 to 50 mC, giving curves of the type shown in Figure 3. For each such curve the corresponding $(\partial \log M / \partial H)_c$ *vs.* H curve was constructed. Then the derivatives at a certain H value were plotted against c, giving curves of the type shown in Figure 4. From the area under this

Table 5

Comparison of h (measured) and h_i (calculated from Equation 9). Concentrations in millimoles/liter.

H mC	h_i mC	h[1] mC	$h_i - h$ mC
	a) $c - 50$ mC		
50.0	53.0		−0.9
45.0	48.5	49.4	−0.9
40.0	44.0	45.3	−1.3
35.0	39.5	41.2	−1.7
30.0	35.4	37.0	−1.6
25.0	31.4	32.9	−1.5
20.0	27.9	28.7	−0.8
15.0	24.6	25.0	−0.4
10.0	22.0	21.6	+0.4
5.0	19.5	18.6	+0.9
H mC	h_i mC	h[2] mC	$h_i - h$ mC
	b) $c = 25$ mC		
50.0	51.2	50.4	0.8
45.0	46.4	44.8	1.6
40.0	41.5	41.1	0.4
35.0	36.6	36.6	0.0
30.0	32.0	32.2	−0.2
25.0	27.5	27.8	−0.3
20.0	23.3	23.4	−0.1
15.0	19.4	19.3	0.1
10.0	15.7	15.1	0.1
5.0	12.8	12.2	0.6

[1] Average of titrations 11 and 12.

[2] Titration 10.

curve, the h value could be calculated that corresponded to H and a certain value of c. In the present work, the lowest H value used in this calculation was 5 mC. For a given value of H, the difference between h and H will increase with increasing c. Any difference between the integrated hydrogen ion concentration, h_i, and h, as measured from the glass electrode, should therefore be more easily detected in the solutions containing most Fe^{3+}. In tables 5 a and 5 b, corresponding to $c = 25$ and $c = 50$ mC, h_i and h may be compared, and as can be seen, the agreement is fairly good.

Let us now discuss the reaction mechanism. The (β, log h) values (Figure 1) corresponding to $c = 0.1$ and $c = 0.2$ mC do not seem to differ significantly, indicating that at these low concentrations mononuclear complexes predominate. Thus bh should be a function of $1/h$ only (see Equation 7). Figure 5 shows a plot of bh against $1/h$. The data can be well represented by a straight line. (Less weight should be given to the points at low $1/h$ values, since in this range the determination of β will be largely influenced by small errors in the redox potentials.) From the intercept and the slope of the line, the following equilibrium constants may be calculated:

$$\varkappa_{11} = 0.90 \pm 0.10 \text{ mC} = (9.0 \pm 1.0) \cdot 10^{-4} \text{ C},$$
$$\varkappa_{12} = 0.49 \pm 0.10 \text{ (mC)}^2 = (4.9 \pm 1.0) \cdot 10^{-7} \text{ C}^2$$

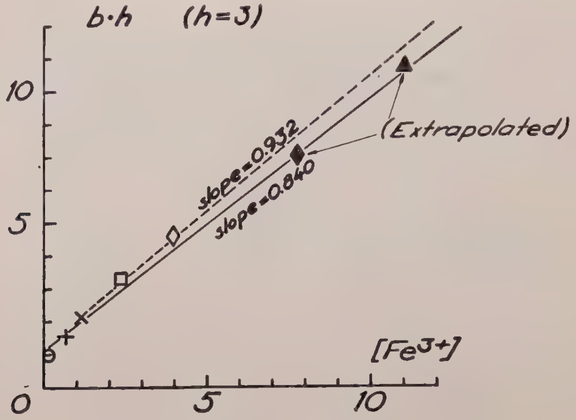


Fig. 6. *bh vs. M* for $h = 3$ mC.

In Figures 6 to 9, bh has been plotted against M for different, constant h values. (The corresponding β values were determined graphically from the intersection of the experimentally determined β *vs.* log h curves and the vertical lines corresponding to certain constant h values. In Figure 6, two points were obtained by extrapolation of the corresponding β *vs.* log h curves.) Here also, the data can be represented by straight lines, indicating that no polynuclear complexes other than dinuclear ones were formed. The intercepts of the straight lines on the bh axis lead to the mononuclear constants (see Equation 7). Since $\varkappa_{12} \cdot h^{-1}$ is very small, it will not add appreciably to the intercept, and the intercepts of all the lines are therefore very much the same, i.e., about $0.9 \approx \varkappa_{11}$, in good agreement with the value obtained above from titrations 1 to 4 (Figure 5). On the other hand, the slopes of the lines differ considerably. We can therefore immediately conclude that $\varkappa_{21}$ (see Equation 7) alone cannot explain the data. But if the slopes are multiplied by the corresponding h values, the following figures are obtained:

$$\begin{array}{ll} h = 3: & 3 \cdot 0.840 = 2.52 \\ h = 5: & 5 \cdot 0.507 = 2.54 \\ h = 8: & 8 \cdot 0.280 = 2.24 \\ h = 15: & 15 \cdot 0.161 = \underline{2.42} \\ & \text{average} \quad 2.43 \end{array}$$

Since these figures do not differ appreciably, and show no trend, we may infer that the predominating dinuclear process is the following (see Equation 7):

$$2\,\mathrm{Fe}^{3+} + 2\,\mathrm{H_2O} \rightleftharpoons \mathrm{Fe_2(OH)_2^{4+}} + 2\,\mathrm{H}^+$$

From Equation 7 we then get

$$\varkappa_{22} = 2.43/2 = 1.22 \pm 0.10 \text{ mC} = (1.22 \pm 0.10) \cdot 10^{-3}\text{ C}$$

At this point the data seem to be exhausted; the experimental results do not allow calculation of more equilibrium constants. (If the two extrapolated points of Figure 6 are not used, the slope of the "best" straight line through the remaining points is 0.932, instead of 0.840. If the slope 0.932 is used the average value of $2\,\varkappa_{22}$ becomes

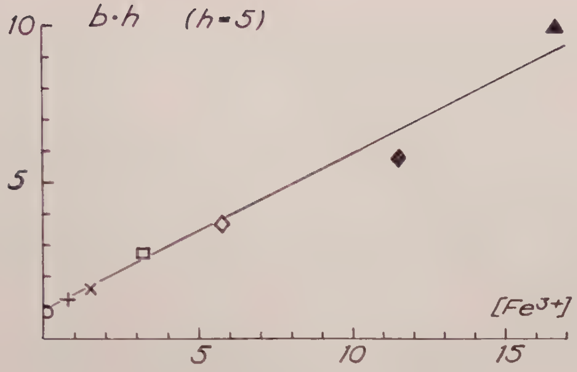


Fig. 7. *bh vs. M* for *h* = 5 mC.

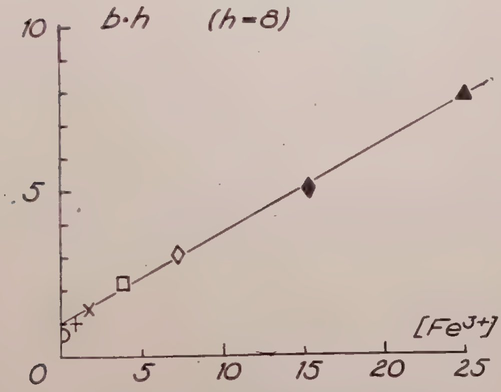


Fig. 8. *bh vs. M* for *h* = 8 mC.

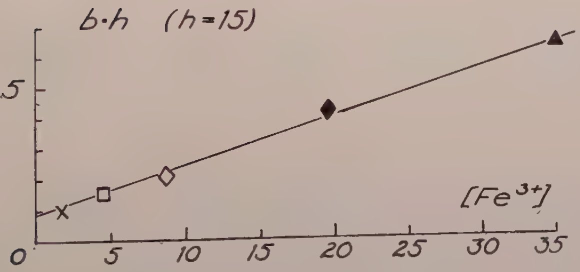


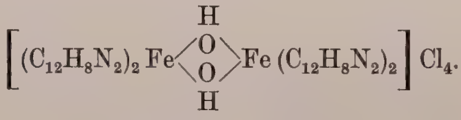
Fig. 9. *bh vs. M* for *h* = 15 mC.

2.50. Since this value agrees closely with that calculated from the slope 0.840 ($3 \cdot 0.840 = 2.52$), the latter slope and thus the two extrapolated points of Figure 6 were used in the final calculation of the average value of $2\ \varkappa_{22}$.)

The curves of Figures 1 and 2 have been calculated from the above values of the equilibrium constants. As can be seen from Figure 1 the experimental points fall close to the calculated curves. Since the Z *vs.* log h plot (Figure 2) is based upon the glass electrode potential and analytical data only, any large errors in the titrations, or in their interpretation, should show up very noticeably in this plot. At c values lower than 5 mC, the accuracy of the measured Z values is very low, since only small amounts of hydrogen ion are liberated in the hydrolysis. In the range $c = 5$ to $c = 50$ mC, the accuracy is greater, and in this range the experimentally determined Z values seem to support the above interpretation of the β *vs.* log h curves.

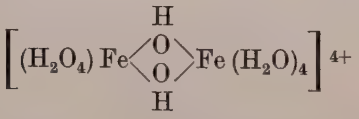
Even if the experimental data show no evidence for the formation of trinuclear etc. complexes, the consideration of such higher complexes may perhaps become necessary at higher iron(III) concentrations or higher pH values than those studied in this investigation.

In the equilibria discussed above, the hydrolysis products have been formulated so as to contain hydroxyl groups. However, the emf measurements alone cannot supply information about the detailed structure of the complexes. Thus the *oxo* complex Fe_2O^{4+} is equally consistent with the measurements as the *diol* complex $Fe_2(OH)_2^{4+}$. On the basis of a comparison between hydrolysis data and ammine complex data, J. Bjerrum (*3*, p. 68) concluded that the existence of the *ol* type of structure is, in general, more plausible than that of the *oxo* type. Gaines, Hammett and Walden (*9*) have prepared a solid phenanthroline complex compound, whose properties are said to correspond to the formula



$$\left[(C_{12}H_8N_2)_2\,\mathrm{Fe}{<}\begin{matrix}\overset{\mathrm{H}}{\mathrm{O}}\\ \underset{\mathrm{H}}{\mathrm{O}}\end{matrix}{>}\mathrm{Fe}\,(C_{12}H_8N_2)_2\right]Cl_4.$$

One may expect the dinuclear complex ion found in the present work to have the same *diol* type of structure:



$$\left[(H_2O_4)\,\mathrm{Fe}{<}\begin{matrix}\overset{\mathrm{H}}{\mathrm{O}}\\ \underset{\mathrm{H}}{\mathrm{O}}\end{matrix}{>}\mathrm{Fe}\,(H_2O)_4\right]^{4+}$$

Pauling (*19*) suggests that Fe-Fe bonds may help in stabilizing complexes of this type; such metal-metal bonds must be assumed to be present in $Fe_2(CO)_9$ to account for the short Fe-Fe distance and the diamagnetism of the compound.

SUMMARY

The hydrolysis of the Fe^{3+} ion has been studied at 25.0° C and $[ClO_4^-] = 3$ C, using emf methods and a titration technique, by which the total concentration of Fe^{3+} (c) was kept constant while the hydrogen ion concentration could be conveniently varied from high values, where the hydrolysis could be neglected, up to the precipitation point of Fe^{3+}. When sodium hydroxide was added to the iron solutions no steady potentials were obtained, due to the formation of a gelatinous precipitate, which dissolved only slowly. Sodium hydrogen carbonate did not give rise to these difficulties, and it was therefore used as the base in all the titration experiments reported.

The hydrogen ion concentration was measured with a glass electrode, and the concentration of free Fe^{3+} ions with a redox (platinum) electrode. The equilibrium

solutions studied contained Fe^{2+}, and no evidence was found for the formation of complexes, containing both Fe^{2+} and Fe^{3+}.

In the concentration range studied, $c = 0.1$ to 50 mC, the data can be explained in terms of the equilibria

	Equilibrium constants
$Fe^{3+} + H_2O \rightleftharpoons FeOH^{2+} + H^+$	$\varkappa_{11} = (9.0 \pm 1.0) \cdot 10^{-4}$ C
$Fe^{3+} + 2H_2O \rightleftharpoons Fe(OH)_2^+ + 2H^+$	$\varkappa_{12} = (4.9 \pm 1.0) \cdot 10^{-7}$ C^2
$2Fe^{3+} + 2H_2O \rightleftharpoons Fe_2(OH)_2^{4+} + 2H^+$	$\varkappa_{22} = (1.22 \pm 0.10) \cdot 10^{-3}$ C.

Some of the hydrogen ion concentrations, as calculated from the glass electrode potentials, were checked by employing Equation 9 (see text), applicable to any set of polynuclear complex equilibria (*13*). (in Equation 9, h denotes the equilibrium concentration of hydrogen ions, H the corresponding stoichiometrical hydrogen ion concentration, and M the equilibrium concentration of metal ions, $[Fe^{3+}]$.)

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Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm.

Symbols used in the text (mC denotes millimoles/liter).

B = stoichiometrical concentration of the base solution used, mC.
b = $1/\beta - 1$, ratio of the fraction metal ion not hydrolyzed to the fraction hydrolyzed, dimensionless.
c = total, stoichiometrical concentration of the hydrolyzed metal ion, mC.
E = measured redox emf, mV.
E' = $E - E_j$, mV.
E_0 = a constant, defined by Equation 2, mV.
E_0' = a constant, defined by Equation 3, mV.
E_g = measured emf of the cell $-$ reference electrode $|H^+|$ glass electrode $+$.
E_{go} = a constant, defined by Equation 1, mV.
E_j = $-0.018\,h$, the liquid junction emf in a cell containing 3000 mC $NaClO_4$ solution in contact with a solution containing h mC hydrogen ions, $(3000-h)$ mC Na^+, and 3000 mC ClO_4^-, mV.
H = stoichiometrical hydrogen ion concentration, mC.
H_M = the stoichometrical hydrogen ion concentration of the acid iron(III), iron(II) solution used, mC.
h = the equilibrium concentration of hydrogen ions, mC.
h_i = the equilibrium hydrogen ion concentration, as calculated from Equation 9.
M = the equilibrium metal ion concentration, mC.
p = the number of metal atoms in the complex $M_pOH_q{}^{(pz-q)+}$, dimensionless.
q = the number of hydroxyl groups in the complex $M_pOH_q{}^{(pz-q)+}$, dimensionless.
v_B = volume of base solution added, ml.
Z = $(h - H)/c$, the average number of hydrogen ions set free per metal ion added, dimensionless.
β = M/c, the fraction of metal ion which is not hydrolyzed, dimensionless.
$\varkappa_{pq}$ = the equilibrium constant for the reaction $pM^{z+} + q\,H_2O \rightleftarrows M_pOH_q{}^{(pz-q)+} + qH^+$.

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A simplified small-scale method for the preparation of chondroitin sulphuric acid from cartilage

By HARRY BOSTRÖM and BENGT MÅNSSON

With 2 figures in the text

In a recent paper from this laboratory (1) STRANDBERG briefly surveyed various methods of preparing chondroitin sulphuric acid and described a method which gave a good yield of a fairly pure product from bovine cartilage. We applied his technique on small amounts of cartilage in our previous studies of the sulphate exchange in chondroitin sulphuric acid (2, 3). We soon found it desirable, however, to use a simpler technique, avoiding time-consuming evaporations, precipitations and centrifugations.

In this paper a simplified technique is described. Its principles are: (a) extraction in 2 per cent sodium hydroxide, (b) neutralization with glacial acetic acid and dialysis followed by (c) deproteinization by passing the samples through a kaolin column under pressure.

Experimental

Method

The cartilage (*e.g.* bovine trachea, joints of foetal calves, costal cartilage of rabbits and rats) is cut in thin slices or minced in a homogenizer. 2–5 g of the material are poured into an Erlenmeyer flask, distilled water added to 25 ml and the mixture boiled for 5 minutes and then cooled to about 0° C in an ice-box. After cooling, 2.5 ml of 5.5 N sodium hydroxide to a final concentration of about 2 per cent are added and the flask is shaken in the cold-room at about + 4° C for 20 hours in a shaking machine. Glacial acetic acid is added to pH 6–7 to the mixture (in the cold). The mixture is filtered on glass-wool and the filtrate is dialyzed in a cellophane bag against running distilled water for 24 hours at room temperature. After evaporation to about 50 ml *in vacuo*, the sample is allowed to pass through a column of kaolin[1] (30 mm high and 25 mm in diameter) in a specially constructed steel tube (200 mm high and 25 mm in inner diameter, Fig. 1) under an air pressure of 2–4 atm. This procedure takes 5–10 hours and after passage an absolutely clear solution is obtained. This is evaporated to thin syrup *in vacuo* and precipitated with 3 volumes of ethanol in the presence of sodium chloride. After drying with absolute ethanol and ether, a light, white powder of the sodium salt of chondroitin sulphuric acid is obtained. The yield amounts to about 30–40 mg of the sodium salt of chondroitin sulphuric acid per gram of fresh cartilage.

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[1] Kaolin, Washed Powder (American Standard), J. T. Baker Chemicals Co, Phillipsburg, N.J.

In order to avoid time-consuming manual work when a whole series of preparations is to be made, the aforementioned technique is further simplified and applied in a manner which permits simultaneous preparation of 10 samples of chondroitin sulphuric acid. After dialysis of all the samples, the 10 cellophane bags are suspended together in a 60 cm high glass vessel and a pre-warmed (40° C) air stream passed from the bottom of the glass vessel overnight. Sufficient water is thereby evaporated. For the passage through kaolin a set of 10 steel tubes (Fig. 2) is used and after the passage the 10 samples are simultaneously and separately concentrated overnight to thin syrup in 150 ml wide-necked Erlenmeyer flasks by blowing pre-warmed air (40° C) into the flasks. The sodium salt of chondroitin sulphuric acid is then precipitated with ethanol as above.

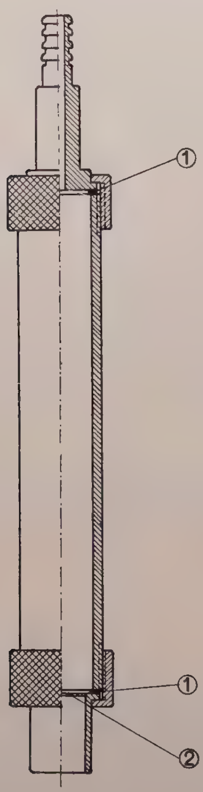

Fig. 1. Schematic picture of the specially constructed steel tube used in deproteinization with kaolin. In the figure, 1 denotes rubber packings and 2 denotes the perforated membrane. The inner diameter of the tube is 25 mm and its height 200 mm.

Preparation of the kaolin column

Kaolin[1] in a 15 per cent suspension is allowed to stand overnight and is then after shaking ready for use. A filter paper is placed on the perforated membrane in the bottom of the steel tube (Fig. 1), the tube is then filled with the 15 per cent kaolin suspension, closed and an air pressure of 2–4 atm. is applied to the tube. About 70 ml

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[1] Kaolin, Washed Powder (American Standard), J. T. Baker Chemicals Co, Phillipsburg, N.J.

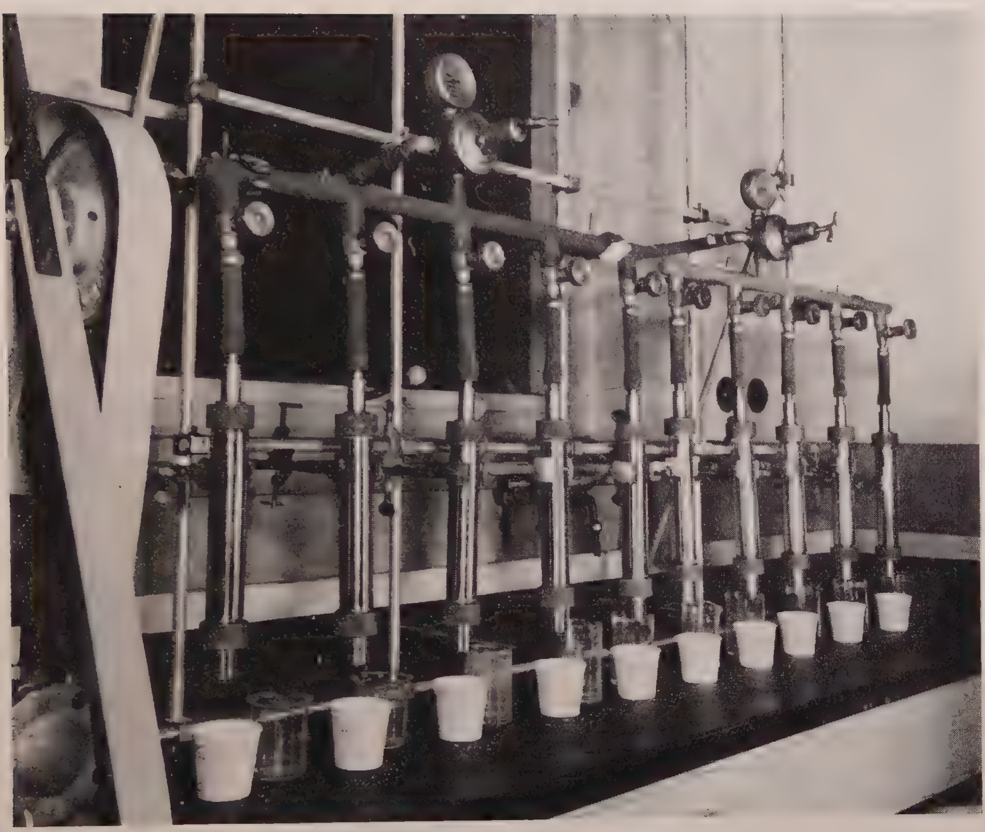

Fig. 2. Photograph showing the whole set of steel tubes used.

of water are allowed to run through. (Care must be taken to avoid drying of the kaolin layer.) The tube is then opened and refilled with the sample to be passed and pressure is again applied until the whole sample has passed through the column.

Results and discussion

Several hundred preparations of chondroitin sulphuric acid have been made according to the method described. The content of nitrogen and ester-bound sulphur and the nitrogen sulphur ratio for three representative series of preparations are recorded in Table I. In order to obtain sufficient material for more complete analyses, 50 mg of each of forty preparations were pooled, dissolved in 25 ml of distilled water, reprecipitated with 3 volumes of ethanol and dried with absolute alcohol and ether. The analytical figures for this pooled sample are given in Table II.

As seen from Tables I and II, chondroitin sulphuric acid with a fairly high degree of purity can be prepared according to the present small-scale method. The nitrogen values are low and the samples are protein free, Biuret-negative. The only deproteinization procedure used is passage of the samples through a 3 cm high kaolin column.

Table I. Analytical figures for three series of the sodium salt of chondroitin sulphuric acid prepared according to the method described.

Series No.	No. of samples	Mean % N of dry substance (Calcd. 2.78)	Mean % S of dry substance (Calcd. 6.37)	Mean N/S ratio (Calcd. 1.00)
10: 52	10	2.79	5.57	1.15
24: 52	10	2.68	5.65	1.09
27: 52	9	2.47	5.52	1.02

Table II. Analytical data for 40 pooled samples of the sodium salt of chondroitin sulphuric acid prepared according to the method described.

	Found	Calculated
Biuret	Negative	
Metachromasia	Positive	
Nitrogen	2.81 %	2.78 %
Sulphur	5.66 %	6.37 %
Galactosamine (identified and detd. acc. to GARDELL (4))	30.4 %	35.60 %
Uronic acid (TRACEY (5))	37.8 %	38.57 %
Specific rotation $[\alpha]_D$	−22.5°	(Meyer and Rapport (6) $[\alpha]_D = -30°$)

With this arrangement the efficiency of the treatment with kaolin is considerably increased.

The analytical figures found for sulphur and ash are somewhat lower than the calculated ones, probably due to the loss of some ester sulphate during the extraction with alkali.

The procedure described may also be useful for preparation of sulpho-mucopolysaccharides from other sources with high contents of these compounds *e.g.* aorta and cornea. In other tissues, however, in which the content of sulpho-mucopolysaccharides is fairly low, *e.g.* skin, sclera and intestinal mucosa, the present technique seems to be of no value.

By increasing the volumes of the pressure tubes and a corresponding increase in the amounts of kaolin, this method allows preparation of chondroitin sulphuric acid from cartilage on an even larger scale. The greatest advantages of the method are, however, its usefulness in metabolic and enzymatic studies or whenever series of small-scale preparations of chondroitin sulphuric acid are desirable.

SUMMARY

A simple small-scale method for the preparation of chondroitin sulphuric acid from cartilage is described. The cartilage is extracted with 2 per cent sodium hydroxide. The extract is neutralized with acetic acid, dialyzed, concentrated and deproteinized by passing through a kaolin column under a pressure of 2–4 atmospheres. The chondroitin sulphuric acid obtained is protein free with a sulphur content close to the theoretical.

The authors are greatly indebted to Professor ERIK JORPES for his valuable advice during the work and to Doctor SVEN GARDELL who kindly performed the identification and estimation of galactosamine.

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Factors influencing the exchange of the sulphate group of the chondroitin sulphuric acid of cartilage in vitro

By Harry Boström and Bengt Månsson

With 7 figures in the text

A number of workers have demonstrated a high sulphate fixation in certain tissues of different species of animals after the administration of S^{35}-labelled sodium sulphate (1, 2, 3, 4, 5). This appears to be due mainly to the sulphate exchange in the esterified mucopolysaccharides such as chondroitin sulphuric acid (6, 7), mucoitin sulphuric acid and heparin (8).

Layton *et al.*, using a tissue culture technique, made a series of studies of the sulphate fixation in a number of tissues (9, 10). They found, for example, a highly differentiated fixation in embryonic chick tissues, and a greater retention in granulation tissue and injured muscle tissue from healing wounds (11), as well as in cartilaginous tumour tissues (12), than in corresponding normal tissues. In other studies, Layton *et al.* demonstrated, in *in vitro* and *in vivo* experiments, a decrease in the sulphate fixation in the tissues after the administration of cortisone (13, 14).

In an earlier investigation, we used a slicing technique in the study of the exchange of the sulphate group in chondroitin sulphuric acid in slices of costal cartilage from suckling calves (15). This method implies isolation of the chondroitin sulphuric acid and definite separation of its sulphate group; it has proved to be useful for the further study of the conditions regulating the exchange of sulphate in the chondroitin sulphuric acid of cartilage.

In the present investigation, this *in vitro* technique has been improved and applied to the study of a number of factors influencing this reaction, such as the age of the experimental animals, the incubation temperature and storage of the cartilage. The effects of some commonly used enzyme inhibitors (16, 17) on the sulphate exchange were studied in a series of experiments. SH inhibitors in particular were found to produce a considerable degree of inhibition (18, 19, 20). Of some interest is the fact that it was possible to demonstrate a strong inhibition by cortisone and a moderate inhibition by salicylic acid.

Experimental

Reagents

1. Krebs-Ringer-bicarbonate solution.[1]
2. Krebs-Ringer-phosphate solution.[1]
3. S^{35}-labelled Krebs-Ringer-bicarbonate solution. To 650 ml of the ordinary Krebs-Ringer-bicarbonate solution (reagent 1) were added 10 mC of carrier-free

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[1] Prepared according to the directions given in W. W. Umbreit, R. H. Burris and J. F. Stauffer: "Manometric Techniques and Tissue Metabolism," Burgess Publishing Co. Minneapolis 15, Minn. 1951, page 119.

S^{35}-labelled sodium sulphate[1] in approximately 0.5 ml of distilled water. The solution was poured into 30 ml flasks, frozen and stored as ice. The reagent was used over a period of 2 to 3 months, the same solution being used in all the experiments described in this paper. Before use, the content of one of the flasks was thawed and used in one or two experiments. The radioactivity of this isotope solution at the beginning of these experiments was of such an order of magnitude that, if 1 ml of this solution was added to 25 ml of reagent 1, the radioactivity of the final solution amounted to 1.8×10^6 c.p.m. per sq.cm. (measured as $BaSO_4$ at infinite thickness). The date of this determination was recorded, and the subsequent determinations of the radioactivity were corrected for decay to this date.

4. The inhibitors used, which were of the highest commercial purity available or laboratory preparations[2], were added in the majority of cases directly to the Krebs-Ringer-bicarbonate solution; in a few cases they were dissolved in small quantities of alcohol. Corrections were made for the inappreciable effect of alcohol on the process studied by means of control experiments with alcohol alone.

Standard method

The costal cartilage from eight newly killed, 1–3 day old calves was freed from muscle, connective tissue and perichondrium and sliced to a thickness of approximately 0.3 mm in a specially constructed slicing machine. The slices were received in a vessel containing Krebs-Ringer-bicarbonate solution. When the slicing was completed, the slices were poured on a Büchner funnel (without filter paper) and the solution sucked off. 5 g of the mixed moist slices were then transferred to each of 20 Erlenmeyer flasks containing 25 ml of the Krebs-Ringer-bicarbonate solution. The flasks had previously been kept in a water bath at 37° C for 30 minutes and aerated with a gas mixture (93.5 per cent O_2 plus 6.5 per cent CO_2). 1 ml of S^{35}-labelled Krebs-Ringer-bicarbonate solution was added to each of the flasks and the time noted. Two of the 20 samples served as controls in each experiment and were subsequently treated as described in the following. The remaining 18 samples were used for experiments under varying conditions, *e.g.* variations in the temperature, variations in the time of incubation and the presence of different inhibitors, as reported under the appropriate headings.

After addition of the isotope, the samples were incubated at 37° C and aerated in an oxygen-carbon dioxide mixture for 8 hours. During this time, the flasks were mechanically rocked to and fro in the water bath in such a way that the path of motion of the bottom of the flasks comprised 45° of a circle with a radius of 30 cm and a rate of 60 movements per minute. Incubation was interrupted by heating the contents of the flask to boiling and then cooling to approximately + 4° C. 2.5 ml of 5.5 *N* NaOH were added to each flask and the flasks shaken in the cold room at about + 4° C overnight. Glacial acetic acid to pH 6–7 was added to the content of each flask, followed by filtration through glass wool into cellophane bags. After dialysis against running distilled water overnight, the sample was evaporated and passed through a kaolin column in a specially constructed steel tube, as described in an earlier paper (21). The solution was then passed through an anion exchange column (15) (Dowex 2, 20–40 mesh) 1 cm in diameter and 30 cm in length. The preparation of the chondroitin sulphuric acid was then completed as described in another paper (21), in which the analytical values and the criteria for the purity of the sodium salt of the chondroitin sulphuric acid obtained were also given.

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[1] Obtained from A.E.R.E., HARWELL, England.

[2] The iodosobenzoate was synthetized and kindly supplied by Dr. Bo SÖRBO. The arsenicals were obtained from the Research Institute of National Defence.

Determination of the radioactivity

90–100 mg of the chondroitin sulphuric acid preparation obtained were hydrolyzed on a boiling water bath with 6 N HCl for 4 hours. The sulphate was then precipitated with $BaCl_2$ and the radioactivity determined after plating (15) in a Geiger-Müller counter with a 2.1 mg per sq.cm. mica end-window Geiger-Müller tube. All the samples had a thickness of 25 mg per sq.cm., which corresponds approximately to infinite thickness.

Reproducibility of the method

As may be inferred from the description of the standard method, the pooled slices of costal cartilage from 7–8 calves killed on the same day and at approximately the same time were used for one experiment. The costal cartilage form another similar group of calves was used for the next experiment, and so on.

Each experiment consisted of the preparation of 20 samples of chondroitin sulphuric acid and determination of their radioactivity. Two of these samples were incubated under standard conditions and the remaining 9 pairs of samples were incubated under varying conditions, depending on the factor to be studied. This implies that two kinds of statistical variations are involved in determining the error of the method, *i.e.*, the variation in the individual experiment and the variation between the different experiments. The former, wich is dependent upon such factors as measuring errors in determining the radioactivity, irregularities in the incubation and in the isolation of chondroitin sulphuric acid, was calculated from the difference between double determinations. *The standard deviation of a single determination was found to be ± 4.6 per cent and ± 3.2 per cent in a double determination.*

The variation between the experiments, wich also includes the variation in the activity of the cartilage from different groups of calves, was calculated from the mean values of the normal samples in all the experiments and was found to be ± 21.6 per cent, thus significantly larger than the variation of a single sample.

In the present investigation, the influence of the variation between the different experiments was eliminated by comparing only those values obtained in one and the same experiment. All the values included in one table or in one curve thus belong to one and the same series. The values given are as a rule mean values of double determinations.

Removal of inorganic sulphates

Particular difficulty was encountered in removing the excess of the isotope-labelled sodium sulphate from the preparation of chondroitin sulphuric acid isolated. Several methods were tried, *e.g.* precipitation with $BaCl_2$, dialysis, repeated washing of the chondroitin sulphuric acid with 80 per cent alcohol, and dialysis after the addition of large quantities of non-labelled sodium sulphate. None of these methods proved to be entirely satisfactory. Finally, the following procedure was adopted.

After dialysis, the solutions of chondroitin sulphuric acid were allowed to pass through an anion exchange column (Dowex 2, 20–40 mesh) 30 cm in length and 1 cm in diameter. After being used for one sample, the column was washed with approximately 0.5 liter of 4 *N* HCl and then with 0.5 liter of distilled water; it could then be used for the next sample. A set of 10 such columns was used. This technique is simple and effectively removes all contaminating sulphate.

Thickness of the slices

With the slicing technique used in this investigation, the costal cartilage is cut into slices with a specially constructed slicing machine. The apparatus is provided with a rotating knife (30 cm in diameter and a rate of 300 r.p.m.). It is constructed on approximately the same principles as a commerical machine for slicing cooked meat. The thickness of the slices can be varied between 0.1 and 1 mm. Experiments with variations of the thickness within these limits showed a thickness of 0.2–0.5 mm to be the most suitable. Thicknesses above and below these values resulted in lower values for the S^{35} incorporation and poorer reproducibility of the results. In the present investigation, all the slices were cut to a thickness of approximately 0.3 mm.

Influence of homogenization of the cartilage

In a series of experiments, 5 g portions of cartilage were homogenized by treatment for 30 seconds, 1 and 2 minutes, respectively, with a homogenizer[1] before incubation with the standard method. As shown in Table I, the ability of the cartilage to incorporate S^{35} was reduced by homogenization.

Table I. Effect on the S^{35} uptake in chondroitin sulphuric acid *in vitro* of treatment of the cartilage in a homogenizer for varying periods. Incubation temperature: 37° C. Time of incubation: 8 hours.

Time of treatment in the homogenizer (minutes)	Inactivation per cent	Comments
1/2	67	Slight homogenization
1	86	Moderate homogenization
2	93	Almost complete homogenization

Influence of different suspending media

The two isotonic salt solutions most frequently used in slicing experiments are Krebs-Ringer-bicarbonate solution and Krebs-Ringer-phosphate solution. The rate of the sulphate exchange in both these solutions was found to be fairly similar; thus both solutions appear to be useful in studies such as the present.

Influence of nitrogen atmosphere

The influence of a nitrogen gas atmosphere on the incorporation of S^{35} was studied by means of the following experiment. Two Erlenmeyer flasks containing Krebs-

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[1] Homogenisator, Edmund Bühler, Tübingen, Germany.

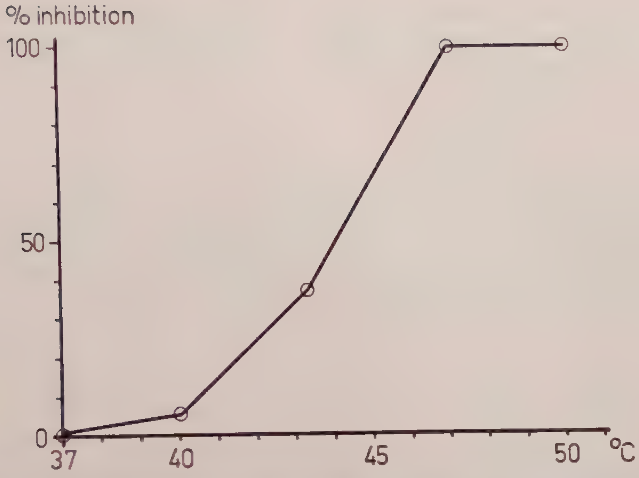

Fig. 1. Inactivation of cartilaginous slices by heat. The curve shows the effect on the sulphate exchange of chondroitin sulphuric acid of pre-treatment of the slices at different temperatures for 30 minutes before incubation was started. Incubation temperature: 37° C. Time of incubation: 8 hours.

Ringer-bicarbonate solution were strongly aerated for 30 minutes with a gas mixture of 93.5 per cent N_2 and 6.5 per cent CO_2. 5 g of cartilage slices were then incubated in each of these flasks, using the standard method in other respects. Two standard tests served as controls. In a nitrogen atmosphere, the S^{35} incorporation was found to amount to only 36 per cent of that under standard conditions.

Inactivation of the slices by increase of temperature

Earlier experiments have shown that no exchange of sulphate took place if the slices of cartilage were boiled before incubation. We wished to ascertain the temperature that is critical for this reaction. A series of samples was therefore pre-treated at varying temperatures for 30 minutes before incubation with the standard method. As seen in Fig. 1, complete inactivation of the slices of cartilage took place at a temperature lying between 43 and 47° C.

Influence of incubation temperature

The rate of sulphate exchange in the chondroitin sulphuric acid of the cartilage is greatly influenced by the temperature at which incubation takes place. In the

Table II. Influence of the incubation temperature on the S^{35} incorporation in chondroitin sulphuric acid *in vitro*. Time of incubation: 8 hours.

Incubation temperature (°C)	Radioactivity counts/min/sq.cm
21	745
29	1 533
37	1 817
50	34

experiments recorded in Table II four different incubation temperatures were tested. As may be deduced from the table, the rate of the sulphate exchange increased until the temperature reached the level (between 43 and 47° C) at which inactivation of the cartilage slices took place.

Inactivation by freezing and thawing the slices

Freezing of the cartilage with subsequent thawing destroys the ability to exchange the sulphate groups. This was demonstrated by the following experiment. One of the samples was frozen rapidly by placing the flask in alcohol at $-40°$ C and keeping it there for two hours. After this time it was thawed cautiously and incubated under standard conditions. No appreciable uptake of S^{35} could be demonstrated in the frozen and subsequently thawed cartilage.

Decrease in activity of the cartilage with time after the slaughter

The ability of the cartilage to incorporate S^{35} decreased with increasing length of time after slaughter. The change in the activity of the cartilage was found be to inappreciable during the first four hours, whereas considerable "devitalization" could be demonstrated 24 and 48 hours, respectively, after the slaughter. After 4 days, only very little activity could be noted. In all the experiments mentioned in this paper, incubation was started 2–4 hours after the animal had been slaughtered.

S^{35} incorporation as a function of the duration of incubation

The relationship between the incorporation of S^{35} in the sulphate group of chondroitin sulphuric acid and the duration of incubation was studied in several experiments. The results of two typical such experiments are recorded in Fig. 2. No incorporation of S^{35} was found in those samples taken at the time 0 and obtained by heating the slices of cartilage to boiling immediately after addition of the isotope. In the other samples incubated according to the standard method, the process was interrupted by boiling at times ranging between 30 minutes and 10 hours. It is seen from the course of the curves that there was a successive increase in the incorporation of S^{35} with an increase in the duration of incubation. The reaction seemed to take place somewhat more rapidly during the 0–2 hour period of time than during the 2–10 hour period.

S^{35} incorporation as a function of the age of the animals

The following experiment was made to ascertain the influence of the age of the experimental animal upon the incorporation of S^{35}. The tracheal cartilage of a suckling calf (1 day old), of a young animal (2 years old) and of an old animal (12 years old) respectively, was prepared, sliced and incubated according to the standard method. The incorporation of S^{35} was found to decrease with rising age. The activity in the cartilage of the young animal and of the old animal amounted to 57 per cent and 26 per cent, respectively, of that in the cartilage of the suckling calf (Table III).

Inhibitors

The effect of a large number of more or less group-specific inhibitors (16, 17) on the incorporation of S^{35} was tested. Varying quantities of the respective inhibitors were added to the Krebs-Ringer-bicarbonate solution containing the slices of car-

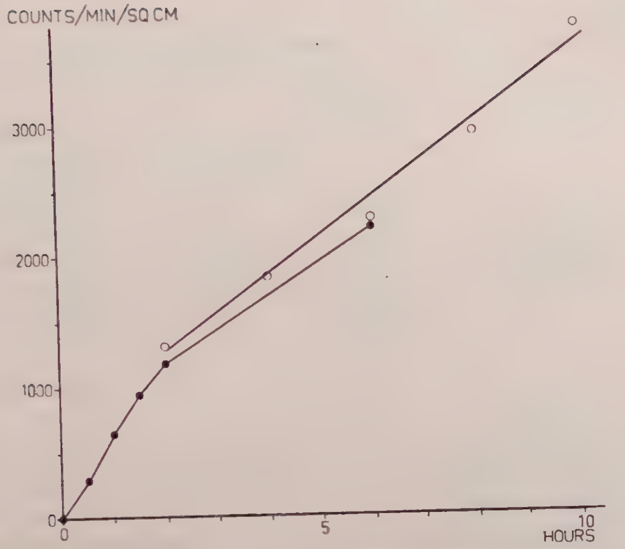

Fig. 2. The S^{35} uptake in the sulphate group of chondroitin sulphuric acid of cartilage *in vitro* as a function of the time of incubation. Incubation temperature: 37° C.

Table III. Influence of age on the S^{35} incorporation in chondroitin sulphuric acid of tracheal cartilage *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

Animal	Approximate age	Radioactivity counts/min/sq.cm
Suckling calf	1 day	2 348
Heifer	2 years	1 329
Old cow	12 »	621

tilage, approximately 30 minutes before addition of the isotope and incubation according to the standard method. The results of the inhibition experiments are recorded in Tables IV–VII and Figs. 3–6. In the tables, the concentration of the inhibitor causing a 50 per cent inhibition is given in those cases in which this figure could be obtained from the curves (Figs. 3–6). In those cases in which only a few concentrations of the inhibitor were tested, the figure is given for the concentration resulting in the degree of inhibition closest to 50 per cent.

Metal enzyme inhibitors

The four metal inhibitors tested, *i.e.*, potassium cyanide, sodium azide, thiourea and 8-hydroxyquinoline, produced relatively slight or moderate inhibition of the process studied (Table IV). As may be inferred from the table, the greatest degree of inhibition was produced by 8-hydroxyquinoline, whereas thiourea had the weakest effect. The inhibitory effect of cyanide on the incorporation of S^{35} has earlier been observed by LAYTON (11).

Table IV. Effect of some different metal enzyme inhibitors on the S^{35} uptake in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

Inhibitor	Concentration of inhibitor M/l	Per cent inhibition
Potassium cyanide	8.0×10^{-3}	53
Sodium azide	8.0×10^{-3}	72
Thiourea	8.0×10^{-3}	12
8-hydroxyquinoline......	8.0×10^{-4}	75

Carbonyl group reagents

The effect of the carbonyl group inhibitors was also relatively slight (Table V). The greatest degree of inhibition was obtained with phenylhydrazine. This observation is of some interest since it has been shown that phenylhydrazine is also able to inactivate sulphydryl enzymes (22). No inhibition was produced by low or moderately high concentrations of dimedon.

Table V. Effect of some different carbonyl group reagents on the S^{35} uptake in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

Inhibitor	Concentration of inhibitor M/l	Per cent inhibition
Hydroxylamine	8.0×10^{-3}	95
Semicarbazide	8.0×10^{-3}	42
Sodiumbisulphite........	8.0×10^{-4}	39
Phenylhydrazine	8.0×10^{-4}	67
Potassium cyanide	8.0×10^{-3}	53
Dimedon	8.0×10^{-3}	0

Sulphydryl reagents

Tests were made with a large number of reagents that are known to be able to react with the SH groups in proteins (18, 19, 20). It is seen from Table VI a that all such reagents tested, which represented different types of SH inhibitors, produced a considerable degree of inhibition. Thus, 50 per cent inhibition was obtained with iodoacetic acid, phenylmercuric nitrate and parachloromercuric benzoate, even in a concentration 100 times lower than that necessary for the corresponding degree of inhibition with potassium cyanide. Only one-thousandth of this concentration was required for the two arsenicals tested, *i.e.*, lewisite and diphenylarsine chloride. The inhibition caused by mercurials and iodosobenzoate could to some extent be prevented by glutathione as shown in Table VI b.

Table VI a. Effect of some SH inhibitors on the S^{35} uptake in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

Inhibitor	Concentration of inhibitor M/l	Per cent inhibition
Iodosobenzoate	4×10^{-4}	50
Iodoacetate	6×10^{-5}	50
Phenylmercuric nitrate	2×10^{-5}	50
p-Chloromercuric benzoate	4×10^{-5}	50
Lewisite	8×10^{-6}	50
Diphenylchloroarsine	8×10^{-6}	50
Sodium arsenite	8.0×10^{-4}	88
Sodium selenite	8.0×10^{-5}	46
Copper chloride	8.0×10^{-4}	79
Chloroacetophenone	5.0×10^{-5}	66

Table VI b. Preventive effect of glutathione on the inhibition of the S^{35} incorporation in chondroitin sulphuric acid *in vitro* of iodosobenzoate and phenylmercuric nitrate. Glutathione was added to the incubation flasks 60 minutes and the inhibitors 30 minutes before the addition of the isotope. Incubation temperature: 37° C. Time of incubation: 8 hours.

Experiment	Inhibitors	Radioactivity counts/min./sq.cm	Per cent inhibition
A	Controls	2 517	
	4 mM glutathione	1 995	21
	0.77 mM iodosobenzoate	78	97
	4 mM glutathione + 0.77 mM iodosobenzoate	1 507	40
B	Controls	3 081	
	4 mM glutathione	2 457	20
	0.13 mM phenylmercuric nitrate	360	88
	4 mM glutathione + 0.13 mM phenylmercuric nitrate	899	71

A more thorough study was made of certain of the SH inhibitors, tests being made with a greater number of different concentrations. These experiments are exemplified in Figs. 3–6, in which the inhibition curves are recorded for iodosobenzoate, iodoacetic acid and the mercurials and arsenicals tested.

Cortisone

Cortisone was among the other substances tested. According to LAYTON's investigations (13, 14) this substance reduces the fixation of S^{35} in various tissues; this observation was confirmed by our experiments. Cortisone, in the form of cortisone alcohol, was dissolved directly in the Krebs-Ringer-bicarbonate solution. It proved to have a strongly inhibiting effect on the sulphate exchange in chondroitin sulphuric acid in vitro. With a concentration of 2.8×10^{-4} M per liter (10 mg per cent), the inhibition amounted to 35 per cent (Table VII and Fig. 7). Of the substances hitherto tested, only the SH reagents proved to be more powerful inhibitors.

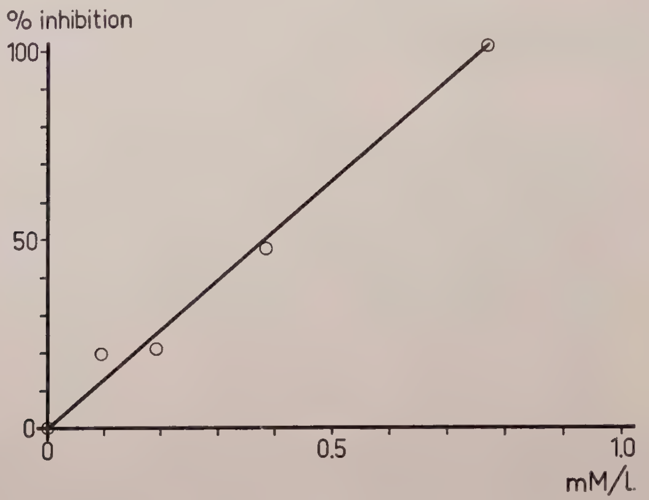

Fig. 3. Effect of different amounts of iodosobenzoate on the S^{35} incorporation in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

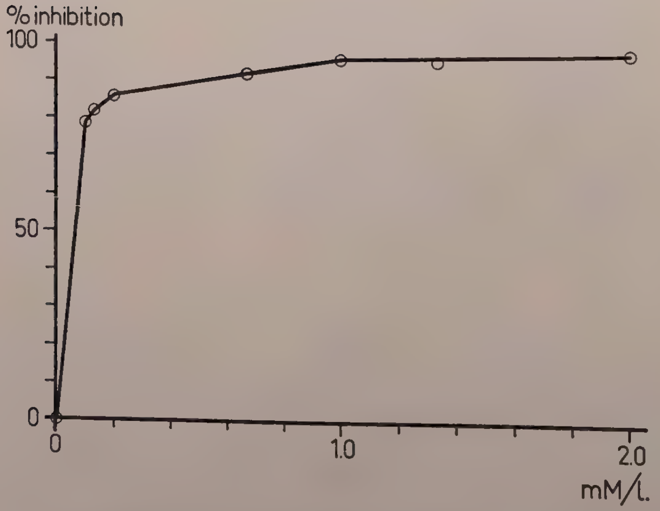

Fig. 4. Effect of different amounts of iodoacetate on the S^{35} incorporation in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

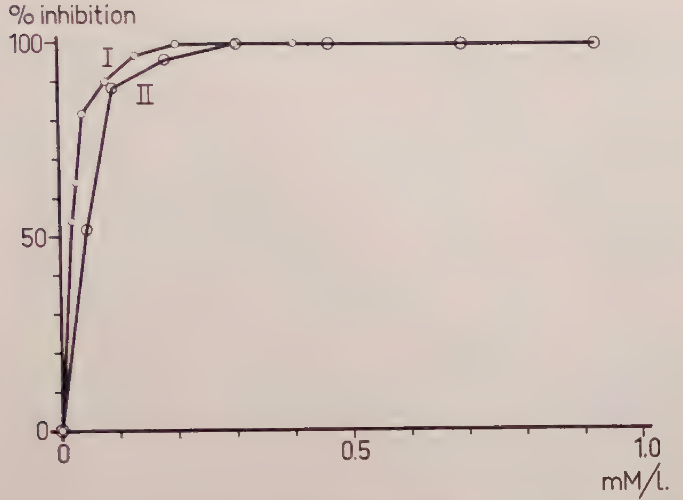

Fig. 5. Effect of mercurials on the S^{35} incorporation in chondroitin sulphuric acid *in vitro*. Curve I represents phenylmercuric nitrate and Curve II represents *p*-chloromercuric benzoate. Incubation temperature: 37° C. Time of incubation: 8 hours.

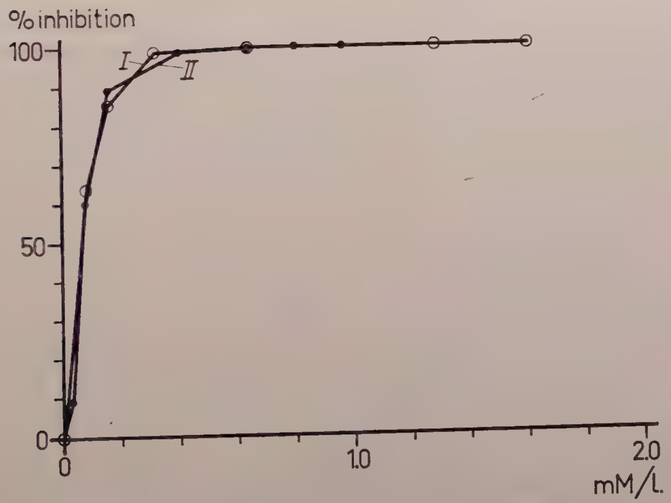

Fig. 6. Effect of arsenicals on the S^{35} incorporation in chondroitin sulphuric acid *in vitro*. Curve I represents diphenylchloroarsine and Curve II represents lewisite. Incubation temperature: 37° C. Time of incubation: 8 hours.

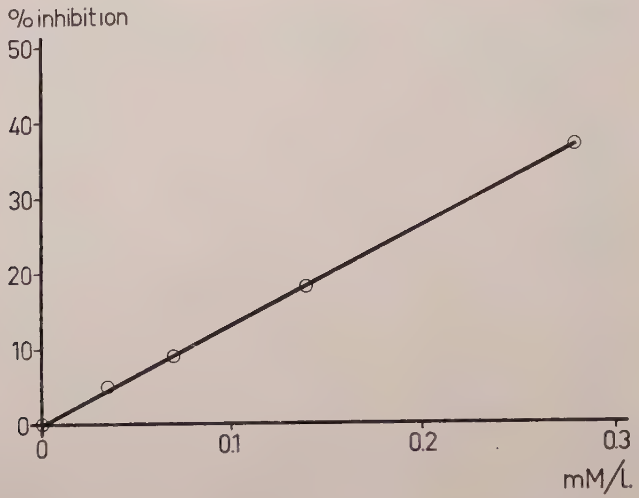

Fig. 7. Effect of cortisone alcohol on the S^{35} incorporation in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

Effect of some other substances

The inhibitory effect of a number of other substances, such as alcohol, toluene, formalin, penicillin, salicylic acid, benzoic acid and *p*-aminobenzoic acid, was also tested. It is evident from Table VII that some of these substances (formalin, toluene and salicylic acid) produced moderate inhibition of the sulphate fixation, whereas others (alcohol, penicillin, benzoic acid and *p*-aminobenzoic acid) had little or no effect.

Table VII. Effect of some different substances on the S^{35} uptake in chondroitin sulphuric acid *in vitro*. Incubation temperature: 37° C. Time of incubation: 8 hours.

Inhibitor	Concentration of inhibitor M/l	Per cent inhibition
Cortisone alcohol	2.8×10^{-4}	36
Salicylic acid	3.8×10^{-3}	30
Sodium benzoate	2.7×10^{-3}	0
p-aminobenzoic acid	2.9×10^{-3}	0
Formalin	8.0×10^{-4}	52
Ethyl alcohol	2.0×10^{-1}	16
Toluene	5.0×10^{-3}	66
Sodium benzylpenicillate	3.8×10^{-3}	0

Discussion

In the present investigations, the slicing technique described in an earlier paper (15) was further elaborated and improved. A number of factors have been found to influence the exchange *in vitro* of the sulphate group of chondroitin sulphuric acid. Among these factors are the thickness of the slices, the incubation temperature, the time elapsing between the slaughter of the experimental animal and the beginning of incubation, and the age of the animal from which the cartilage is taken. Good reproducibility of the results was obtained by keeping these factors as far as possible constant, and by eliminating the greatest source of error, *i.e.*, contamination of the chondroitin sulphuric acid by labelled inorganic sulphates, by means of a special technique. The use of the present *in vitro* method was greatly facilitated by the simultaneous elaboration of a simplified technique for the small-scale preparation of chondroitin sulphuric acid from cartilage (21). This technique combined with the procedures used in the present investigations appears to be useful not only in the study of the sulphate exchange in chondroitin sulphuric acid, but also in the study of the metabolism of the chondroitin sulphuric acid in general.

In vitro experiments with the present method demonstrated a considerable exchange of sulphate in the chondroitin sulphuric acid of slices of cartilage. Many details in the process studied indicate that the sulphate exchange is catalyzed by one or several enzymes. Thus, for example, the activity is destroyed by heating, it is influenced by the incubation temperature, it is destroyed by storage of the cartilage, and is inhibited by various substances in low concentrations. On the other hand, no noteworthy uptake of S^{35} took place in 24 hours when sodium sulphate labelled with S^{35} was added to pure chondroitin sulphuric acid alone (15). All these facts favour the opinion that the sulphate exchange is regulated by enzymatic systems, possibly a specific chondrosulphatase present in the cartilage. However, all attempts to separate the activity from the living cells have hitherto been unsuccessful. The destruction of the activity in the cartilage caused by freezing and thawing and by homogenization is probably due to the disintegration of the cellular structure of the slices. Further, the marked decrease in the rate of sulphate exchange when the ordinary oxygen-carbon dioxide gas mixture is replaced by a nitrogen-carbon dioxide gas mixture seems to indicate that oxygen is required for the process in question. The fact that in the present experiments some exchange of sulphate also took place in a "nitrogen atmosphere" was presumably due to the incomplete exclusion of oxygen.

It was demonstrated that the rate of the sulphate exchange in the chondroitin sulphuric acid of cartilage from cattle decreases with rising age. This is in agreement with the observations recently reported by LAYTON *et al.* (23), *i.e.*, that the rate of fixation of S^{35} in certain tissues in the mouse decreases with rising age.

The results of the inhibition experiments showed that the incorporation of S^{35} in the slices of cartilage is apparently of a relatively complex nature, since it is inhibited by a number of substances. On the other hand, these experiments showed that the process is particularly sensitive to the action of SH reagents.

Obviously, until a pure enzyme system has been obtained, these inhibition experiments do not permit the conclusion that the specific enzyme system regulating the sulphate exchange is an SH enzyme. They do, however, indicate that enzymes are present in the slices of cartilage that are particularly sensitive to SH reagents and which influence the sulphate exchange in chondroitin sulphuric acid.

The inhibition of the S^{35} incorporation in cartilage caused by cortisone, demonstrated *in vitro* and *in vivo* (24), is of particular interest and was earlier observed by LAYTON (13, 14). As shown in our experiments, the sulphate exchange in chondroitin sulphuric acid is inhibited by relatively weak solutions of cortisone. Thus, of all the substances tested by us, only the SH reagents were found to have a more powerful inhibitory action on this process.

The effect of cortisone upon the sulphate exchange in chondroitin sulphuric acid that has been demonstrated does not, however, reply to the important question of whether or not cortisone decreases the rate of metabolism in the whole molecule of chondroitin sulphuric acid. Nor may any definite conclusions be drawn from the investigations made hitherto with regard to a possible relationship between the effect of cortisone studied and its effect upon various mesenchymal diseases.

The effect of salicylic acid is remarkable, but no definite conclusions regarding the nature of this effect are permissible until further studies have been made.

SUMMARY

An *in vitro* technique used earlier in studies of the sulphate exchange in the chondroitin sulphuric acid of cartilage has been further elaborated and improved. The costal cartilage from newly slaughtered calves is cut into thin slices and incubated in a Krebs-Ringer-bicarbonate solution containing S^{35}-labelled sodium sulphate. The chondroitin sulphuric acid is isolated from the slices of cartilage and the radioactivity in its sulphate group is determined after hydrolysis.

This method has been used to study the effect of certain factors influencing the sulphate exchange in chondroitin sulphuric acid *in vitro*. This process has been found to be influenced by, for example, the age of the experimental animal, the incubation temperature and the presence of oxygen. The exchange of sulphate is found to be completely inhibited if the cartilage is heated to 43–47° C, is homogenized or is frozen and subsequently thawed. The process is inhibited by a considerable number of substances, of which SH reagents are found to have the most powerful effect. Strong inhibition has also been obtained with cortisone, and moderate inhibition with salicylic acid.

The authors are greatly indebted to Professor ERIK JORPES for his constant interest and encouragement and to Dr. LEONARD GOLDBERG, for his valuable advice regarding the statistical calculations.

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ARKIV FÖR KEMI Band 6 nr 4

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The influence of cortisone upon the sulphate exchange of chondroitin sulphuric acid

By H. Boström and E. Odeblad

With 2 figures in the text

Ragan and co-workers (1) showed that cortisone inhibited the formation of granulation tissue in wounds by delaying the development of all the elements of connective tissue. Layton (2, 3, 4) using S^{35}-labelled sodium sulphate as the tracer substance, noted in *in vitro* and *in vivo* studies a decrease in the sulphate-fixation capacity of different tissues after the administration of cortisone; he suggested the cause to be an inhibitory effect of cortisone on the synthesis of chondroitin sulphate or of sulphomucopolysaccharides.

A report is given in the present paper of experiments similar to those of Layton concerning the sulphate exchange of the chondroitin sulphuric acid of cartilage. As it seemed to be of interest in experiments of this kind to isolate the substances to be studied, methods (5, 6, 7) were used which allowed isolation of the chondroitin sulphuric acid. A simple comparative autoradiographic study on the sulphate fixation in the skin of normal rats and cortisone-treated rats, respectively, was also made according to a technique used in previous investigations (8, 9).

Experimental

In vivo experiments. Two groups of white adult rats weighing about 250 g, each group containing 20 animals, were used. The rats in one of these groups were given daily subcutaneous injections of 1 mg of cortisone acetate[1] per 100 g of body weight. The rats in the other group served as controls. On the fifth day of cortisone administration the rats in both groups were injected intraperitoneally with S^{35}-labelled "carrier-free" sodium sulphate[2] (150 μC in 0.1 ml of distilled water per 100 g of body weight). 48 hours later all the animals were sacrificed. The costal cartilage of the rats in each group was dissected out and pooled, as were small samples of skin (0.5 × 0.5 cm) taken from the back of each rat. The material thus obtained was treated as follows.

Chondroitin sulphuric acid was isolated from the pooled cartilage of each group according to a method described elsewhere (7). The two preparations of the sodium salt of chondroitin sulphuric acid thus obtained were hydrolyzed with 6 *N* HCl for 24 hours. The sulphate was precipitated as $BaSO_4$. The samples were plated as previously described (6) and the radioactivity was measured by means of a Geiger-Müller counter with a 2.1 mg/sq.cm mica end-window Geiger-Müller tube. The values of the measurements were expressed as counts/min./sq.cm at infinite thickness.

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[1] Cortone, Merck and Co. Inc., Rahway, N.J. U.S.A.

[2] Obtained from A.E.R.E., Harwell, England.

The pooled pieces of skin were fixed in methanol for 24 hours. They were then passed through isopropanol (for 3 hours) and xylene (for 3 hours), embedded in paraffin and cut into 10 μ thick sections. These were mounted on metacrylate slides and deparaffinized in xylene and ethanol. Contrast autoradiography was then employed, using Gevaert Dentus rapid film. The time of exposure was 24 days. The film was developed for 30 minutes at + 18.3° C in Kodak DK 20 with bromide added to 0.2 per cent.

In vitro experiments. Slices of costal cartilage from 5 young calves were prepared as described in a previous paper (6). About 5 g of the slices were put into each of ten 150 ml Erlenmeyer flasks placed in a 37° C water bath. The flasks contained 25 ml of Krebs-Ringer-bicarbonate solution in which different amounts of cortisone alcohol[1] had been dissolved. Thus, 2 of the flasks contained 10 mg % of cortisone alcohol and the other pairs of flasks 5, 2.5, and 1.25 mg %, respectively. The last pair of flasks, serving as controls, contained no cortisone. After 30 minutes, 1 ml of an S^{35}-labelled Krebs-Ringer-bicarbonate solution[2] was added and the flasks were incubated for 8 hours in the water bath as described elsewhere. The reaction was then stopped by boiling the contents of the flasks. Chondroitin sulphuric acid was isolated from the slices of cartilage and the radioactivity of the ester sulphate was measured as previously described (7).

Results

The results of the *in vivo* experiments are recorded in Table I and Fig. 1. As seen from the table, the radioactivity of the sample of chondroitin sulphuric acid obtained from the cartilage of the cortisone-treated group amounted to only about 60 per cent of that of the control group.

The autoradiographs seen in Fig. 1 show a considerably higher S^{35} content in the skin of rats in the control group than in that of the cortisone-treated group.

The curve in Fig. 2 represents the results of an *in vitro* experiment; when the concentration of cortisone alcohol in the medium was increased, a corresponding decrease was noted in the rate of the sulphate exchange in the chondroitin sulphuric acid. A cortisone alcohol concentration of 10 mg % led to about 35 per cent inhibition.

Table I. Analytical figures and results of radioactivity measurements of the sodium salt of chondroitin sulphuric acid prepared from the costal cartilage of a control group and a cortisone-treated group of rats. The animals were sacrificed 48 hours after the intraperitoneal adminstration of a tracer dose of S^{35}-labelled sodium sulphate.

Group	Per cent of air-dry substance		N/S ratio	Radioactivity counts/min./sq.cm (inf. thickness)
	Nitrogen	Sulphur		
Control group	2.32	4.54	1.17	34 290
Cortisone-treated group .	2.42	4.83	1.15	19 410

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[1] Kindly supplied by Merck and Co. Inc., Rahway. N.J. U.S.A.

[2] 10 mC "carrier-free" Na_2SO_4 obtained from A.E.R.E., Harwell, England, dissolved in 650 ml of Krebs-Ringer-bicarbonate solution.

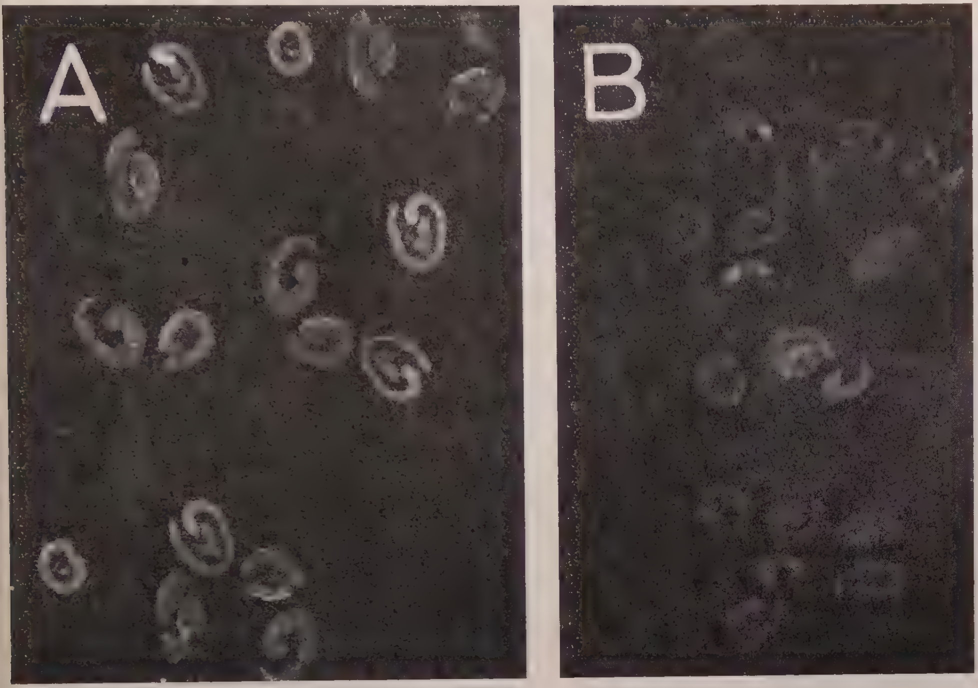

Fig. 1. Autoradiographs of pieces of skin obtained from the same two groups of rats showing a considerably higher S^{35} content in the control group (A) than in the cortisone-treated group (B).

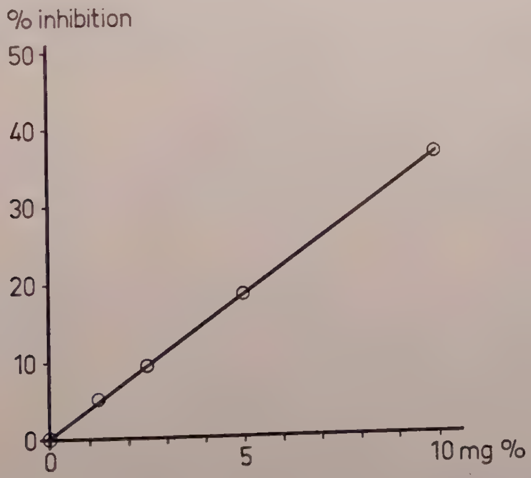

Fig. 2. Influence of different amounts of cortisone alcohol on the sulphate exchange in chondroitin sulphuric acid of the costal cartilage of calves as studied *in vitro*.

Discussion

The results obtained confirm and complement the findings of LAYTON. *In vivo*, a decrease was demonstrated in the sulphate fixation of the skin following cortisone administration. With regard to the cartilage, *in vivo* and *in vitro* experiments showed that a considerably smaller amount of S^{35} given as sodium sulphate was incorporated in the sulphate group of chondroitin sulphuric acid than under normal conditions. The technique used, including isolation and identification of chondroitin sulphuric acid from the cartilage, removal of all traces of inorganic sulphates by passing the samples of chondroitin sulphuric acid through an anion exchange column (6), splitting off the ester sulphate group by acid hydrolysis and precipitating it as $BaSO_4$ before the radioactivity measurements, seems to exclude most of the sources of error.

The interesting question whether or not the decrease in the S^{35} incorporation in the tissues following cortisone administration reflects an actual inhibition of the synthesis of chondroitin sulphate has previously been discussed by LAYTON (2, 3, 4). In our opinion, the results of the *in vitro* experiments published by LAYTON and those obtained in the present investigation seem to show a decrease in the rate of renewal of the sulphate group of chondroitin sulphuric acid after the administration of cortisone. From the chemical point of view, however, nothing is as yet known about the rate of renewal of other parts of the molecule, *e.g.* the amino group, the acetyl group or the whole carbon skeleton. From the experiments on the sulphate exchange only no conclusions can be drawn with regard to the synthesis *in vitro* of the whole molecule of chondroitin sulphuric acid. Nor do the *in vivo* experiments undertaken permit any conclusions with regard to this synthesis. They only show a decrease in the incorporation of S^{35} in the chondroitin sulphuric acid, probably due to a decrease in the rate of renewal of the sulphate group of this compound caused by cortisone.

SUMMARY

The effect of cortisone on the incorporation of S^{35}-labelled sodium sulphate in the sulphate group of chondroitin sulphuric acid of cartilage was studied in *in vitro* and *in vivo* experiments. It was found that cortisone exhibited a retarding effect on the exchange of the ester sulphate group.

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Chemical and autoradiographic studies on the sulphate exchange in sulpho-mucopolysaccharides

By Harry Boström

With 1 figure in the text

The present dissertation is a summary of fourteen papers dealing with the following topics:

Chapter I

Sulphate exchange in the chondroitin sulphuric acid of the cartilage

I. Boström, H.: On the metabolism of the sulfate group of chondroitinsulfuric acid. J. Biol. Chem. *196*, 477, 1952.

II. Boström, H. and Åqvist, S. E. G.: Utilization of S^{35}-labelled sodium sulphate in the synthesis of chondroitin sulphuric acid, taurine, methionine and cystine. Acta Chem. Scand. *6*, 1557, 1952.

III. Boström, H., Odeblad, E. and Friberg, U.: A quantitative autoradiographic study of the incorporation of S^{35} in tracheal cartilage. Arch. of Biochem. and Biophys. *38*, 283, 1952.

IV. Boström, H. and Månsson, B.: On the enzymatic exchange of the sulfate group of chondroitinsulfuric acid in slices of cartilage. J. Biol. Chem. *196*, 483, 1952.

V. Boström, H. and Månsson, B.: A simplified small-scale method for preparation of chondroitin sulphuric acid from cartilage. Arkiv f. Kemi *6*, 17, 1953.

VI. Boström, H. and Månsson, B.: Factors influencing the exchange of the sulphate group of the chondroitin sulphuric acid of the cartilage *in vitro*. Arkiv f. Kemi *6*, 23, 1953.

VII. Boström, H. and Odeblad, E.: The influence of cortisone upon the sulphate exchange of chondroitin sulphuric acid. Arkiv f. Kemi *6*, 39, 1953.

Chapter II

Sulphate exchange in the sulpho-mucopolysaccharides of the skin

VIII. Boström, H. and Gardell, S.: Uptake of sulphates in mucopolysaccharides esterified with sulphuric acid in the skin of adult rats after intraperitoneal injection of S^{35}-labelled sodium sulphate. Acta Chem. Scand. *7*, 216, 1953.

IX. Boström, H., Odeblad, E. and Friberg, U.: A qualitative and quantitative autoradiographic study on the uptake of S^{35}-labelled sodium sulphate in the skin of the adult rat. Acta Pathol. et Microbiol. Scand. *33*, Fasc. 4, 1953.

X. Jorpes, E., Odeblad, E. and Boström, H.: An autoradiographic study on the uptake of S^{35}-labelled sodium sulphate in the mast cells. Acta Haematol. *9*, 273, 1953.

Chapter III

Sulphate exchange of the sulpho-mucopolysaccharides in different tissues as studied by autoradiography

XI. Odeblad, E. and Boström, H.: An autoradiographic study of the incorporation of S^{35}-labeled sodium sulfate in different organs of adult rats and rabbits. Acta Pathol. et Microbiol. Scand. *31*, 339, 1952.

XII. Odeblad, E. and Boström, H.: A quantitative autoradiographic study on the uptake of labelled sulphate in the aorta of the rabbit. Acta Chem. Scand. *7*, 233, 1953.

XIII. Boström, H. and Odeblad, E.: Autoradiographic observations on the uptake of S^{35}-labelled sodium sulphate in the nervous system of the adult rat. Acta Psychiatr. et Neurol. Scand. *28*, Fasc. 1, 1953.

XIV. Boström, H. and Odeblad, E.: Autoradiographic observations on the incorporation of S^{35}-labeled sodium sulfate in the rabbit fetus. Anat. Record *115*, Fasc. 3, 1953.

Reference will be made to these papers using the Roman figures as listed in the aforegoing.

Introduction

Historical

Among the acid mucopolysaccharides, those esterified with sulphuric acid, the sulpho-mucopolysaccharides, constitute an important group with a wide distribution in different organs and tissues. Thus, chondroitin sulphuric acid is one of the main constituents of the hyaline cartilage. It is also present in the aortic wall, in tendons, sclera, and the dermal connective tissue. Mucoitin sulphuric acid is claimed to occur in the gastric mucosa (1). Hyaluronosulphate (2) is also stated to be present in the cornea. Lately, heparin (3, 4) has been found to be a specific mucopolysaccharide produced by the mast cells. The mucopolysaccharides evidently play an important role in different biological processes, but the information regarding their metabolism is very meagre.

The studies summarized in this treatise deal with some aspects of the metabolism of the mucopolysaccharides. They are, however, confined to questions concerning the fate of the ester sulphate of the sulpho-mucopolysaccharides. The sulphate exchange of the sulpho-mucopolysaccharides has been followed in *in vitro* and *in vivo* experiments with chemical and autoradiographic methods, using S^{35}-labelled sodium sulphate as the tracer substance.

The isotope used in these studies, S^{35}, is a β-emitter which exhibits radiation properties very similar to those of C^{14} and has a half-life time amounting to 87 days. It is fairly suitable for this purpose and has found a wide application in different kinds of tracer studies (5, 6), a few of which have been undertaken with autoradiography (7).

The problems concerning the distribution and excretion of S^{35} after the administration of S^{35}-labelled sodium sulphate, the only form in which S^{35} was used in the present studies, have been dealt with previously by other workers (8, 9, 10, 11). Thus Dziewiatkowski 1949 found a very rapid excretion in the urine and faeces of S^{35} given in the form of sodium sulphate. About 95 per cent was found to have been eliminated by these routes 120 hours after the injection of S^{35} and a rapid fall was noted in the isotope concentration in the blood, liver and brain (10).

In contrast to the rapid fall observed in these tissues, the bone, bone marrow and cartilage showed a high uptake and a slow elimination (10, 12). Dziewiatkowski suggested that the sulphate was incorporated mainly into the chondroitin sulphuric acid of the cartilage (12). He also attempted (1951) to isolate this labelled chondroitin sulphate from the cartilage after adding large amounts of non-labelled chondroitin sulphuric acid as the carrier (13). In a later paper he also studied by means of contrast autoradiography the S^{35} incorporation in the articular cartilage and bone of suckling rats following the injection of labelled sodium sulphate (14). Similar investigations were reported at the same time by Campbell, using the track autoradiographic technique (15).

Layton *et al.* 1949 elaborated a tissue culture technique (16, 17) and used it to study the sulphate fixation in several tissues. They found a high degree of differentiation of the S^{35} uptake in embryonic chick tissue and a considerably higher sulphate fixation in granulation tissue, injured muscle tissue (18) and cartilaginous tumour tissue (19) than in normal tissues. They also demonstrated the utilization of maternal sulphate by mammalian embryos and suckling young (20). In other papers Layton 1951 reported that administration of cortisone decreased the sulphate fixation of different tissues *in vitro* and *in vivo* and suggested that this phenomenon was due to a retarded synthesis of chondroitin sulphate caused by cortisone (21, 22).

Other tracer experiments with S^{35}-labelled sulphate showed that sulphate sulphur was utilized in the synthesis of ethereal sulphates (11, 23). On the other hand it was shown by Tarver and Smith that sulphate sulphur is not incorporated in cystine (24).

The present investigations

The aim of the experiments reviewed here on the sulphate exchange of the sulpho-mucopolysaccharides, with particular reference to the chondroitin sulphuric acid of the cartilage, was:

1) To study the incorporation of S^{35} in chondroitin sulphuric acid of cartilage and skin by administering the S^{35}-labelled sulphate.
2) To study the rate of renewal of the sulphate group of the sulpho-mucopolysaccharides in some tissues.
3) To obtain information about the factors influencing the sulphate exchange of the chondroitin sulphuric acid of cartilage.
4) To obtain an idea of the S^{35} incorporation in other sulphur-containing compounds present in the tissues after the administration of labelled sulphate.
5) To ascertain whether or not autoradiographic visualization of S^{35} would be useful in the studies on the sulphate exchange of sulpho-mucopolysaccharides.
6) To study, by means of autoradiographic visualization of S^{35}, the sulphate fixation in different normal organs and tissues.

The results of these investigations have been published in Papers I–XIV. The summary of these is given below in three chapters.

Chapter I (I–VII) deals with the sulphate metabolism of the cartilage. S^{35}-labelled sodium sulphate was injected intraperitoneally into rats. The incorporation of S^{35} in the ester sulphate group of the chondroitin sulphuric acid was followed for 16 days with a chemical (I) and a quantitative autoradiographic technique (III). In Paper II, the rate of incorporation of S^{35} in the chondroitin sulphuric acid of the cartilage was compared with that in methionine, cystine and taurine, isolated from the liver.

Papers IV–VII describe *in vitro* experiments on the ester sulphate exchange of cartilage. It was found (IV) that S^{35}-labelled sodium sulphate was incorporated in the chondroitin sulphuric acid even in slices of fresh costal cartilage maintained *in vitro* in a Krebs-Ringer-bicarbonate solution. The slicing technique was improved (VI) and with this improved technique the influence of factors such as the temperature, the age, group-specific inhibitors and cortisone (VI, VII) upon the sulphate exchange was studied. Paper V deals with a simplified small-scale method for the preparation of chondroitin sulphuric acid from cartilage, which greatly facilitated the *in vitro* experiments.

Chapter II (VIII–X) deals with a report of the sulphate exchange of the sulpho-mucopolysaccharides of the skin of rats. A chemical (VIII) and a quantitative autoradiographic (IX) study on the incorporation and elimination of S^{35} in the skin was undertaken and a stripping film autoradiographic investigation was made on the S^{35} incorporation in the mast cells of the subcutis (X).

Chapter III is a summary of the experiments published in Papers XI–XIV on the sulphate fixation in various tissues and organs as studied by autoradiography. Some tissues belonging to the gastrointestinal system (XI), the cardiovascular system (XI, XII), the nervous system (XIII) and certain embryonic tissues (XIV) were investigated with this technique.

CHAPTER I

Sulphate exchange in the chondroitin sulphuric acid of cartilage

A. In vivo experiments

Adult white rats were given intraperitoneal injections of S^{35}-labelled sodium sulphate. Nine groups of animals, each comprising twenty rats, were used. The groups of animals were sacrificed at different times after the injection, the first group after 2 hours and the last after 16 days. The blood of all the animals in a group was pooled, as were the ribs with the intercostal muscles and the livers. Chondroitin sulphuric acid was prepared from the costal cartilage by applying on a small scale the method of JORPES (25) as modified by STRANDBERG (26). The different preparations of chondroitin sulphuric acid obtained, the analytical figures of which are given in Paper I, Table I, were hydrolyzed in hydrochloric acid and the sulphate precipitated as $BaSO_4$ for measurements of the radioactivity. The inorganic sulphate of the intercostal muscles was extracted and precipitated as $BaSO_4$ and the total sulphur of the blood was transformed into sulphate by oxidizing with sodium peroxide before the precipitation as $BaSO_4$ (I).

Taurine was isolated from the lipid material of the pooled livers of each group. This compound, which follows the phospholipid fraction, was set free by hydrolysis with hydrochloric acid and separated on a cation exchange resin column (Dowex 50). Using the same cation exchange resin, methionine and cystine were isolated after acid hydrolysis of proteins insoluble in trichloroacetic acid.

After checking the identity of taurine, methionine and cystine by paper chromatography, the samples were treated with sodium peroxide and the sulphate precipitated as $BaSO_4$ (II).

The different samples of $BaSO_4$ originating from the sulphate sulphur of chondroitin sulphuric acid of cartilage, the inorganic sulphate of intercostal muscles, the total sulphur of the blood and of the cystine, methionine and taurine from the livers, were plated and the radioactivity measured by means of a Geiger-Müller counter.

In a similar experiment (III) 9 adult white rats were given injections of S^{35}-labelled sodium sulphate. The animals were sacrificed at the same intervals of time after the injection as those in the previous experiment. In this case, a piece of trachea was removed from each animal and fixed in methanol. The specimens were embedded in paraffin and sections were taken in the usual way. They were then mounted on metacrylate slides and deparaffinized in xylene. Contrast autoradiography was performed using an X-ray film, which was developed according to ODEBLAD (27). The autoradiographs obtained were subjected to qualitative and quantitative examinations. The quantitative estimations of the density of the autoradiographs corresponding to the relative amount of S^{35} fixed in the sections were performed with a recording photometer as described earlier by ODEBLAD (27).

S^{35} incorporation in chondroitin sulphuric acid

A high S^{35} incorporation could be demonstrated by splitting off the ester sulphate group of the chondroitin sulphuric acid isolated from the cartilage and measuring its radioactivity. These findings confirm the previous suggestions of DZIEWIATKOWSKI and co-workers (12, 13). As also shown by DZIEWIATKOWSKI (14) and CAMPBELL (15) the S^{35} uptake in the cartilage is easily visualized by means of autoradiography (III, Fig. 1).

S^{35} content of other sulphur-containing compounds

An extremely rapid decrease in the radioactivity of the free inorganic sulphates of the intercostal muscles was noted (I, Fig. 1, Curve II). Although it was impossible to isolate sufficient amounts of inorganic sulphates from the cartilage for radioactivity measurements, it may be suggested that a somewhat similar rapid decrease in the S^{35} content of the inorganic sulphates also takes place in this tissue.

Of the other compounds studied, taurine showed a very low S^{35} incorporation, while in methionine there was no significant uptake (II). As shown earlier by TARVER and SMITH (24), the present studies also show no significant incorporation of S^{35} in cystine. As also noted by DZIEWIATKOWSKI (12) the radioactivity of the total sulphur of the blood exhibited a rapid fall, ultimately attaining a low value (I, Fig. 1, Curve I). These findings support the view that S^{35} administered as sulphate is only to a very small extent incorporated in compounds other than ester sulphates.

Rate of renewal of the ester sulphate group of chondroitin sulphuric acid of cartilage

The uptake of S^{35} in the sulphate group of chondroitin sulphuric acid was followed for 16 days. A maximal uptake was found 24 hours after the injection of labelled sulphate, after which the curve showed a slow even fall during the whole observation period (I, Fig. 1, Curve III). The approximate biological half-life time was estimated by extrapolation to 16 days. The shape of this curve, which was obtained by chemical isolation of the ester sulphate group, was compared with that of the curve (III, Fig. 2) which represents the results obtained by quantitative autoradiographic estimations of the relative S^{35} content of the tracheal cartilage. There was no similarity between the curves up to 24 hours but after that there was a fairly good agreement up to a period of 16 days (Fig. 1), thereby suggesting that the main part of the S^{35} present in the cartilage later than 24–48 hours occurs in the sulphate group of the chondroitin sulphuric acid. The high peak noted in the first part of the curve based on autoradiography is probably due to the high content of labelled inorganic sulphates during the first hours after the injection.

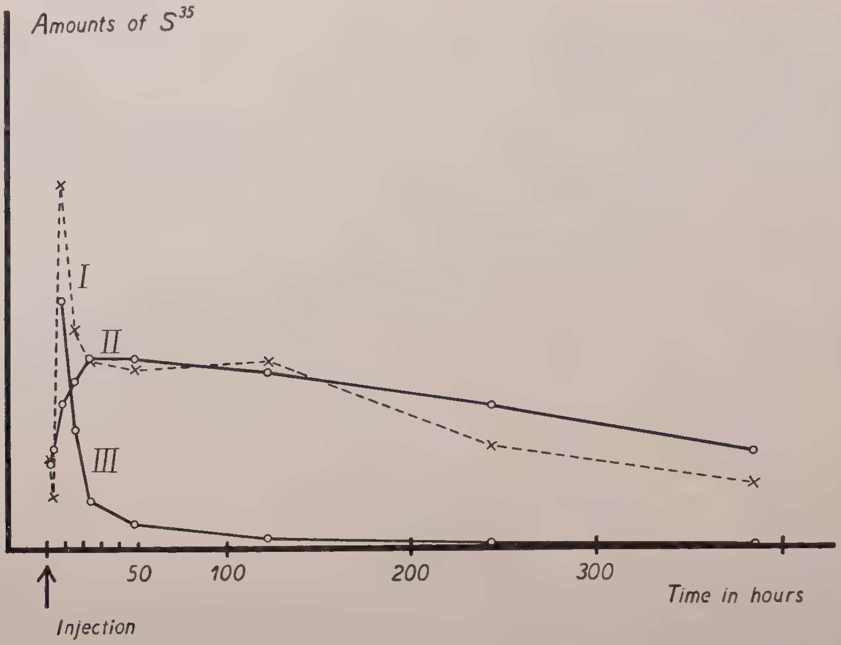

Fig. 1. The sulphate exchange of cartilage after the administration of S^{35}-labelled sodium sulphate in the adult rat. Curve I represents the radioactivity of cartilage as estimated by quantitative autoradiography (Paper III). Curve II represents the radioactivity of the ester sulphate of isolated chondroitin sulphuric acid as estimated in a Geiger-Müller counter (Paper I). Curve III shows the radioactivity of the total sulphur of blood (Paper I).

B. In vitro experiments

As a further tool in the present studies on the sulphate exchange of the chondroitin sulphuric acid of cartilage, a slicing technique was elaborated (IV) and further improved in a later investigation (VI). Fresh costal cartilage from newly killed suckling calves was cut into thin slices. They were incubated in a Krebs-Ringer-bicarbonate solution containing S^{35}-labelled sodium sulphate at a temperature of 37° C in the presence of a gas mixture of oxygen and carbon dioxide. The reaction was stopped by boiling and chondroitin sulphuric acid was isolated from the slices. After removal of contaminating inorganic sulphates, the radioactivity of the ester sulphate of chondroitin sulphuric acid was measured.

Preparation of chondroitin sulphuric acid

In the first of the present studies on chondroitin sulphuric acid (I, II, IV) this substance was isolated according to the technique of JORPES (25) as modified by STRANDBERG (26). It was soon found desirable, however, to use a simpler technique in order to avoid time-consuming evaporations, precipitations and centrifugations. A simplified method was finally evolved (V) and this was found to be extremely useful whenever series of small-scale preparations of chondroitin sulphuric acid from cartilage were desirable. The main principles of this technique are: (a) extraction in 2 per cent sodium hydroxide, (b) neutralization with glacial acetic acid and dialysis followed by (c) deproteinization by passing the samples through a kaolin column under pressure.

Removal of inorganic sulphates

In the experiments mentioned above, the removal of the last traces of labelled inorganic sulphates contaminating the preparations of chondroitin sulphuric acid presented a somewhat difficult problem. Several methods were tried, such as precipitation with $BaCl_2$, repeated washings with 80 per cent ethanol, dialysis in the presence of an excess of non-labelled sulphate. None of those methods proved to be completely satisfactory. The object was finally achieved by passing the samples through an anion exchange resin column (IV, VI).

The S^{35} incorporation in the slices

The results of these experiments showed that considerable amounts of S^{35} were fixed in the slices of cartilage. This observation agrees with the findings of LAYTON and co-workers (17, 18, 19) who demonstrated a sulphate fixation in different tissues by means of a tissue culture technique. The isolation of chondroitin sulphuric acid and splitting off its sulphate group by hydrolysis showed that the S^{35} fixed was present as the ester sulphate. No corresponding S^{35} incorporation took place in 24 hours when sodium sulphate labelled with S^{35} was added to pure chondroitin sulphuric acid alone (IV).

The S^{35} incorporation in chondroitin sulphuric acid increased with the time of incubation. During the first two hours of incubation, the S^{35} incorporation seemed to be more rapid than subsequently. In the interval 2 hours–24 hours, there seemed to be a linear relationship between the incorporation of S^{35} and the time of incubation (IV, VI).

The ability of the cartilage to incorporate sulphate ions remained fairly constant during the first 10 hours after the slaughter, if the material was kept in an ice-box at a temperature of ÷ 4° C. After that time a slow reduction in activity was noticed and on the 4th day only very little activity remained in the cartilage. On the other hand, if fresh cartilage was frozen and then thawed before incubation, no S^{35} uptake occurred in the slices; this agrees with the findings of Layton and co-workers (18). Heating of the slices to 47° C and also homogenization in a small blender destroyed the activity (VI).

The influence of some other factors on the S^{35} incorporation in cartilage was also investigated. Thus, a considerably higher uptake was noted in slices of medium thickness (0.25–0.50 mm) than in thin and thick slices, as well as an increase in the S^{35} incorporation with an increase in the temperature until heat inactivation occurred. It was further found that the activity of the slices was higher in Krebs-Ringer-bicarbonate solution and that a considerably slower uptake took place when the bottles were aerated by a nitrogen-carbon dioxide mixture instead of the ordinary oxygen-carbon dioxide mixture (VI).

Inhibition of the S^{35} incorporation

In order to obtain more information on the process leading to incorporation of sulphate in the cartilage, the effect of several more or less "group-specific" enzyme inhibitors (28, 29, 30) was systematically studied in *in vitro* experiments. Most of the reagents employed were dissolved directly in Krebs-Ringer-bicarbonate solution; the acid reagents were brought to pH 7.4 before use. A few water-insoluble reagents were dissolved in ethanol, which in small amounts had no significant effect on the process to be studied. The inhibitors were added to the slices suspended in Krebs-Ringer-bicarbonate solution half an hour before addition of the isotope.

The results of the experiments presented in Paper VI, Tables IV–VII, showed that a moderate degree of inhibition was brought about by many of the substances, *e.g.* hydroxylamine, semicarbazide, potassium cyanide, sodium azide, thiourea, and formalin. Among the inhibitors tested, however, the SH inhibitors (31, 32, 33) such as arsenicals, mercurials, iodosobenzoate, moniodoacetic acid, copper ions and selenite were found to be the most effective. The inhibition caused by mercurials and iodosobenzoate could to some extent be prevented by glutathione as shown in Paper VI, Table VI b.

C. Influence of cortisone on the S^{35} incorporation

Layton (21, 22) noted a decrease in the sulphate-fixing capacity of different tissues after the administration of cortisone.These observations were confirmed and supplemented by a study on the cartilage (VII).

In an *in vivo* experiment, the animals in one of two groups of rats were treated with a moderate amount of cortisone for seven days. The animals in the untreated group served as controls. On the fifth day the rats in both groups were given injections of S^{35}-labelled sodium sulphate. 48 hours later, the animals were sacrificed and chondroitin sulphuric acid was prepared from the pooled costal cartilage of each group and the radioactivity of the ester sulphate group was measured. As seen in Paper VII, Table I, the S^{35} content of chondroitin sulphuric acid obtained from the cortisone-treated group amounted to only about 65 per cent of that of the control group.

The same phenomenon could be demonstrated *in vitro*. Different amounts of cortisone alcohol were dissolved in Krebs-Ringer-bicarbonate solution containing S^{35}-labelled sodium sulphate and the effect on the S^{35} incorporation was estimated by means of the ordinary slicing technique. As seen in Paper VII, Fig. 2, increasing amounts of cortisone caused an increasing degree of inhibition of the S^{35} uptake in the ester sulphate group of chondroitin sulphuric acid.

Discussion

In these investigations, as in the previous studies by Dziewiatkowski *et al.* (12, 13) and Layton *et al.* (19) the incorporation in cartilage of S^{35} administered as sulphate was demonstrated. The supposition made by these workers that the sulphate fixed in cartilage and other mesenchymal tissues occurs as the ester sulphate in chondroitin sulphuric acid has been confirmed through the isolation by the present author of this substance and the estimation of the radioactivity of its ester sulphate group.

Comparative measurements of the radioactivity of other sulphur-containing fractions, such as inorganic sulphates of muscles, total sulphur of blood, methionine, cystine and taurine sulphur and the demonstration of the low S^{35} content in these fractions 24–48 hours after the injection support the view that S^{35} administered as sulphate is mainly retained in the tissues in ester sulphate compounds. This fact is of certain interest in discussing the value of autoradiography as a tool in sulphate exchange studies. (See Chapter III.)

Another interesting question which arises from studies on the S^{35} incorporation in cartilage is whether or not the S^{35} synthesis demonstrated may be considered to indicate the rate of synthesis of the whole molecule of chondroitin sulphuric acid (21). In my opinion, conclusions must be limited to the sulphate exchange of the chondroitin sulphuric acid. Nothing is as yet known about the rate of renewal of other parts of the molecule, of the amino group, the acetyl group or the whole carbon skeleton. Therefore, experiments on the sulphate exchange only do not permit any inferences with regard to the synthesis of the whole molecule of the chondroitin sulphuric acid.

Many details in the process studied indicate that the sulphate fixation is caused by the action of enzymes. Thus, the activity is destroyed by heating and freezing, it is influenced by the incubation temperature, seems to require the presence of oxygen and is inhibited by various substances. All these facts favour the opinion that the sulphate exchange is regulated by enzymatic systems, possibly a specific chondrosulphatase present in the cartilage. However, all attempts to separate the activity from the living cells have hitherto been unsuccessful. Until a pure enzyme system is obtained, the results found in the inhibition experiments do not permit the conclusion that the specific enzyme regulating the sulphate exchange belongs to the SH enzymes. What is shown is that enzyme systems extremely sensitive to SH reagents seem to be present in slices of cartilage, and that these enzyme systems influence the sulphate exchange. In addition to SH reagents, the sulphate exchange is strongly inhibited by cortisone. This observation is of particular interest because, as pointed out by LAYTON (21), some relationship may exist between the inhibition of the sulphate exchange of chondroitin sulphuric acid and the action of cortisone in collagen diseases, and from the same point of view the moderate inhibition by salicylic acid also seems to be interesting (VI, Table VII).

CHAPTER II

Sulphate exchange in sulpho-mucopolysaccharides of the skin

Chemical studies

The sulphate exchange of the chondroitin sulphuric acid of the skin was investigated in Paper VIII. The skins of the same rats distributed in the same 9 groups as in the aforementioned study on the cartilage (page 46) were removed; the pooled skins in each group were washed, ground and dried with acetone. The total sulphur in one part of the dry powder was oxidized with sodium peroxide and the sulphates precipitated with $BaCl_2$ collected; from another part of the dry powder the preformed ester sulphates and the inorganic sulphate were obtained after acid hydrolysis.

A sulpho-mucopolysaccharide fraction was further isolated from the acetone-dry powder after digestion with pancreatic and intestinal enzymes and subsequent treatment with Lloyd's reagent. The preparation obtained was subjected to electrophoresis in Hyflo Super-Cel as described by GARDELL, GORDON and ÅQVIST (34). With this technique two components were isolated: one contained galactosamine, ester sulphate and all the radioactivity; it was suggested to be chondroitin sulphuric acid. The other component contained glucosamine but no ester sulphate and had no radioactivity; it was suggested to be hyaluronic acid.

The ester sulphate of the sulpho-mucopolysaccharide fraction was isolated after acid hydrolysis and precipitation with $BaCl_2$. Determinations were made of the radioactivity of all the samples, including those obtained after direct hydrolysis of the acetone-dry powder and after oxidation of the organic sulphur.

Autoradiographic studies

In another experiment (IX) four rats from the same litter were given injections of S^{35}-labelled sodium sulphate. At different intervals of time a piece of skin was excised from the dorsal area of each rat. All the specimens were subjected to autoradiography using X-ray film as previously described (page 47). The autoradiographs finally obtained were compared with histological sections stained with haematoxylin and eosin, toluidine blue and according to the Hotchkiss technique (35).

The relative amounts of S^{35} fixed in the corium layer of the skin were estimated by means of quantitative autoradiography. Grain countings in the autoradiographs were performed with the immersion lens and by means of an eye-piece square-net micrometer. The fog grain number was subtracted from the grain number in the area corresponding to the corium, and the sensitometric function expressing the relationship between grain number and exposure was obtained from an empirical curve.

S^{35} incorporation in dermal chondroitin sulphuric acid

As in the case of cartilage, a high S^{35} uptake was found in the skin. Isolation of a sulpho-mucopolysaccharide fraction, which was shown to be a mixture of chondroitin sulphuric acid and hyaluronic acid, and subsequent isolation of the ester sulphate of this fraction disclosed that a high S^{35} incorporation had also occurred in the sulphate group of dermal chondroitin sulphuric acid. In fact, the S^{35} incorporation at this site was higher than in the chondroitin sulphuric acid of cartilage.

Rate of renewal of the sulphate group of dermal chondroitin sulphuric acid

The incorporation and elimination of S^{35} in the ester sulphate group were followed for 16 days. As shown in Paper VIII, Fig. 2, a maximum uptake was noted about 24 hours after the injection. After this time the curve decreased somewhat faster than the corresponding curve for cartilage (I, Fig. 1, and VIII, Fig. 3). The approximate biological half-life time of the sulphate group of chondroitin sulphuric acid in skin as estimated from the curve in Paper VIII, Fig. 2 amounts to 8–9 days. A fairly good agreement (IX, Fig. 3) was found when this curve was compared to the curve showing the relative amount of S^{35} in the corium layer of the skin as estimated by means of quantitative autoradiography.

S^{35} incorporation in other compounds of the skin

Contrary to the high uptake of S^{35} in chondroitin sulphuric acid was the low S^{35} uptake found in the total sulphur fraction, showing that there was a very low S^{35} uptake in most other sulphur-containing compounds of the skin (VIII, Curve I, Fig. 2). An extremely rapid decrease in the radioactivity of the inorganic sulphate fraction takes place, as demonstrated by Curve II in the same figure. This curve, which represents a summation of the curves of free sulphates and of ester sulphates, showed a rapid fall. After 24 hours it reached a considerably lower level than that of the curve for the ester sulphate of the isolated chondroitin sulphuric acid, indicating that the radioactivity of inorganic sulphates after this time is low.

S^{35} incorporation in some different structures of the skin

Corium. As shown by autoradiography (IX, Fig. 2) the corium showed a smooth distribution of S^{35}, interrupted by the pictures of the hair follicles and small vessels. The S^{35} uptake of this layer seems to be mainly due to the incorporation of radiosulphur in chondroitin sulphuric acid. This conclusion may be drawn from the shape of the two curves shown in Paper IX, Fig. 3, indicating a fairly good agreement in radioactivity of the ester sulphate group of chondroitin sulphuric acid and the increase and decrease in the relative amount of S^{35} in the corium layer.

Hair follicles. A rapid uptake and elimination of S^{35} — probably localized in the sheaths of the hair follicles — was found, with a probable maximum 8–16 hours after the injection and a half value reached after some days (IX, Fig. 2). The nature of the sulphur-containing compounds present in the hair follicles is not known. It might, however, be dependent on the presence in the sheaths of an unknown ester sulphate compound with a sulphate exchange more rapid than that of chondroitin sulphuric acid in cartilage and skin.

The incorporation of S^{35} in the *small vessels* is probably due to the incorporation of S^{35} in chondroitin sulphuric acid.

S^{35} incorporation in the mast cells

In the autoradiographic study on the skin (IX) in which X-ray film was used, it was noted that small spots occurred in the autoradiographs. In number, size and localization they corresponded to *mast cells.* With the autoradiographic technique in this investigation, the resolution was, however, too low to permit more careful studies of the mast cells. In order to obtain a higher resolution, the S^{35} uptake of the mast cells was subjected to a particular study (X) by means of stripping film autoradiography (36).

Four young rats were given fairly large doses of S^{35}-labelled sodium sulphate. Pieces of skin were removed after 6 and 18 hours and 1, 2, 5, and 18 days. After fixing in methanol, 4 μ thick paraffin sections were taken in the ordinary way; they were mounted on glass slides and deparaffinized in xylene and ethanol. The sections were covered with stripping film in the ordinary way by lifting the slides underneath the film floating in distilled water. After exposure and development autoradiographs were obtained; their resolution was calculated and estimated to about 8 μ, permitting a study of the mast cells.

The density corresponding to the mast cells was estimated subjectively. This was sufficient to determine the approximate point of maximal storage of S^{35} and to provide some information on the rate of exchange of S^{35} in the mast cells.

In all pictures, the mast cells were clearly visible due to their high content of S^{35}, typical shape and mode of distribution (X, Fig. 1). The estimation of density showed a peak of S^{35} 48 hours after the injection of radiosulphate, followed by a fairly slow decrease in density. Thus, 18 days after injection (X, Figs. 2 and 3) the density had failed to reach half the maximum value.

The remarkably high S^{35} uptake demonstrated in the mast cells after a single injection of S^{35}-labelled sodium sulphate is suggested to be due to an exchange of the ester sulphate groups of heparin or its precursors.

Influence of cortisone on the S^{35} incorporation in the skin

A decrease in the S^{35} uptake similar to that in the cartilage after the administration of cortisone (page 50) was also found in the skin, which is in agreement with the results of LAYTON (21, 22). In the autoradiographic picture (VII, Fig. 1) considerably less S^{35} is seen to be retained in the skin of cortisone-treated rats then of the control rats 48 hours after an injection of S^{35}-labelled sulphate. Most of the S^{35} in the skin at this time after the injection seemed to occur in chondroitin sulphuric acid (VIII, Fig. 2). Therefore, the difference in the S^{35} content found probably indicates a decrease in the rate of the sulphate exchange of chondroitin sulphuric acid in the skin caused by cortisone.

Discussion

These experiments showed that the S^{35} incorporation which takes place in the skin is actually due to an incorporation of S^{35} as ester sulphate in chondroitin sulphuric acid, as suggested by other authors (21, 37). The isotope content of the skin polysaccharide reaches a considerably higher level than that of the cartilage. This may be due to a more rapid exchange of fluid in the skin than in the cartilage.

As demonstrated in Chapters I and II, cortisone seems to reduce the speed of the sulphate exchange in chondroitin sulphuric acid in cartilage and skin. It remains to be ascertained whether or not this phenomenon is common to all the sulpho-mucopolysaccharides in all those tissues in which these compounds are present, *e.g.* tendons, aorta, cornea, sclera and gastric mucosa.

CHAPTER III

Sulphate exchange of sulpho-mucopolysaccharides in different tissues as studied by autoradiography

Introduction

If the results of the experiments reviewed in Chapters I and II and the results reported in the literature (page 44) are summarized and somewhat generalized it appears possible to make the following statements: (a) After the administration of labelled sulphate, the S^{35} incorporation in esterified compounds, *e.g.* sulpho-mucopolysaccharides, is high; (b) an extremely low S^{35} uptake occurs in most other sulphur-containing compounds; (c) the radioactivity of the inorganic sulphates decreases rapidly and shows low values 24–48 hours after the administration of a tracer dose of labelled sulphate; (d) in the skin and cartilage, a good agreement is found between the increase and decrease in the relative amount of S^{35} as estimated by quantitative autoradiography, and the incorporation and elimination of S^{35} in the ester sulphate of chondroitin sulphuric acid as studied by chemical methods, if specimens taken later than 24–48 hours after the injection of the isotope are analyzed.

On the basis of these statements, it may be suggested that the autoradiographic demonstration of a considerable retention of S^{35} in an animal tissue probably indicates the presence in this tissue of a sulpho-mucopolysaccharide, or of some other esterified compound. The autoradiographic technique therefore may be regarded as a more or less specific *in vivo* "staining", visualizing these compounds. Moreover, quantitative autoradiography should be of value in the study of the sulphate exchange in sulpho-mucopolysaccharides in those tissues in which the quantity of these substances is insufficient for chemical isolation.

The experiments reported in Papers XI XIV exemplify the use of qualitative and quantitative autoradiography. The experimental animals consisted throughout of rats or rabbits and the ordinary X-ray film autoradiographic technique (29) was used (page 47).

S^{35} incorporation in different tissues and organs of adult rats and rabbits

Cardiovascular tract. The S^{35} incorporation in the *muscles of the heart* was found to be fairly low or moderate, in accordance with the findings of DZIEWIATKOWSKI and LAYTON (17, 37). The substances in which S^{35} occurs in the muscles are as yet unknown. As suggested by LAYTON (17) S^{35} may be present in esterified compounds in the intercellular substance.

The *aorta*, which is known to contain chondroitin sulphuric acid (1) shows a very high S^{35} uptake (XI, Fig. 8, and XII, Fig. 1). A quantitative autoradiographic study of the incorporation and elimination of S^{35} in the rabbit aorta was undertaken. As seen from the curve in Paper XII, Fig. 2, a maximal accumulation of S^{35} seemed to occur after about two days, and two weeks after the injection about half of this value could be noted. The curve presumably reflects the renewal of the sulphate group in the chondroitin sulphuric acid present in the aortic wall.

In the *spleen* (XI, Fig. 5) a diffuse moderate incorporation of S^{35} occurred, the nature of which is unknown.

Respiratory tract. As previously mentioned (page 47) the chondroitin sulphuric acid in the *tracheal cartilage* showed a rapid sulphate exchange. A moderate S^{35} uptake was noted in the *tracheal epithelium*, possibly due to incorporation in the mucus. In the *lung* a moderate uptake was obtained in the *respiratory epithelium* and an extremely low uptake in the *lymphatic follicles*. A high incorporation, on the contrary, was noted in the *cartilage plates of the lung* (XI, Table III and Fig. 9).

Gastrointestinal tract. The *epithelial layers* of the gastrointestinal tract (distal part of *oesophagus, fundus ventriculi, duodenum*, distal part of *ileum* (XI, Figs. 1–4) and *descending colon*[1]) showed a very high S^{35} uptake, most of the radiosulphur seeming to appear in the mucus. In the gastric mucosa the S^{35} probably occurred in mucoitin sulphuric acid and it was suggested that in other parts of the gastrointestinal tract as well, S^{35} was incorporated in similar sulpho-mucopolysaccharides of the mucus. The isolation of polysaccharide fractions containing ester sulphate from mucus obtained from the ileum of pigs (38) seems to support this view. As, however, the intestinal tract is known to be the main extrahepatic site of phenol conjugation (39) the possibility that some of the S^{35} present in intestinal mucosa occurs as ethereal sulphates cannot be ruled out.

In the *liver* and the *pancreas* a diffuse low or moderate uptake of S^{35} was noted.

Nervous system. In the nervous system a locally differentiated but fairly low S^{35} incorporation was demonstrated (XIII, Figs. 1–4). In general, a considerably higher uptake was found in the gray than in the white matter in all nervous tissues investigated. In the *cerebrum* a particularly high uptake was noted in a cortical layer consisting of polymorphous and fusiform cells. In the *cerebellar cortex* the molecular layer and the Purkinje cells showed a moderate uptake. In the granular layer of the *cerebellum*, on the contrary, a considerable S^{35} uptake was noted. Finally, in the *choroid plexus*, there was a very high S^{35} incorporation.

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[1] ODEBLAD, E. and BOSTRÖM, H. Unpublished observation.

It is not known which chemical substances in the brain retain the sulphate. In analogy to the conditions shown to apply to the incorporation of S^{35} in other tissues, it is not improbable that the S^{35} in the brain may occur in esterified compounds, *e.g.* sulpholipids. The higher degree of sulphate fixation demonstrated, *e.g.* in the granular layer of the cerebellar cortex may, however, also be due to the abundance of cell nuclei in these structures, since the cell nuclei of several tissues seem to fix more sulphate than the protoplasm (40).

The extremely high uptake of sulphate in the *choroid plexus*, finally, is in agreement with the previously demonstrated high sulphate fixation in vascular and epithelial structures. The possibility that some part of the S^{35} present in the choroid plexus occurs as inorganic sulphates cannot, however, be ruled out.

Eye. The presence of sulpho-mucopolysaccharides in the *cornea* and *sclera* is well known. A very high S^{35} uptake was also found in these tissues (XI, Fig. 10). On the other hand, no S^{35} incorporation was noted in the *vitreous body* with its hyaluronic acid.

Kidney. A larger amount of S^{35} was retained in the cortical zone than in the medullary substance of the kidney (XI, Fig. 7). In the cortex most of the S^{35} was retained in the peripheral tubules. In the glomerular and the capsular epithelium of the glomerulus there was a medium uptake. Because an excretion of free and conjugated sulphates is known to take place in the kidney, it may be suggested that most of the S^{35} in the kidney occurs in these compounds.

As shown in studies (41, 42, 43) not included in this treatise on the male and female genitourinary system, the S^{35} incorporation varied considerably with different tissues of these organs.

S^{35} incorporation in the embryonal tissues of the rabbit

According to the findings of LAYTON and co-workers (20) maternal sulphate is utilized by mammalian embryos. In order to study the S^{35} distribution in embryonal tissues, rabbit foetuses, about 29 mm in length, of which the mother was sacrificed 16 hours after having received an injection of S^{35}-labelled sodium sulphate, were subjected to autoradiographic examination (XIV). Well-differentiated autoradiographs were obtained, indicating that the S^{35} incorporation varied markedly in different tissues (XIV, Figs. 1–8, Table I). Thus a very high uptake was noted in all *cartilage structures* (Figs. 1–4). A *calcification centre* in the *vertebral column* appeared as an area with a low uptake surrounded by a border of higher uptake (Fig. 2). In the *aorta* (Fig. 4) a considerable fixation of the isotope was noted and other tissues, *e.g.* foetal *lungs* (Fig. 8), *intestines* (Fig. 7), *tongue*, *dental papilla* of a presumptive tooth (Fig. 5), *cardiac valves* (Fig. 6), also showed a fairly high S^{35} incorporation. On the other hand, no or very little S^{35} was fixed in *e.g.* the *liver* and the *brain*.

On the whole the mode of distribution of S^{35} as found in these foetuses seemed to be somewhat similar to that in adult animals.

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The crystal structure of $CeOSO_4H_2O$

By GEORG LUNDGREN

With 8 figures in the text

In this Institute the crystal structures of basic salts of tetravalent metals are being investigated, and the structures of some thorium and uranium(IV) compounds have been published recently (LUNDGREN and SILLÉN 1949, LUNDGREN 1950, 1952, 1953). In this connection attempts were made to prepare cerium(IV) oxy sulphates. With the methods described in GMELIN-KRAUT's Handbuch (1932) no crystals of sufficient size were obtained, but by hydrothermal hydrolysis of cerium(IV) sulphate solutions of different strengths and acidities two or three new compounds could be prepared, mostly as very small crystals. One of them, however, obtained at about 200° C gave very good crystals and was chosen as a suitable object for a crystal structure investigation. Its formula proved to be $CeOSO_4H_2O$, and the present paper presents the structure determination of this compound.

Preparation of the crystals

A solution was prepared by dissolving about 25 g cerium(IV) sulphate (B. D. H. low in other rare earths) in 200 ml 1 *M* sulphuric acid. 50 ml of this solution were then sealed in a thick-walled glass tube and heated to 200° C in an oven. After a week the tube was cooled and the precipitate filtered off. It consisted of rodshaped yellow crystals of $CeOSO_4H_2O$, 0.05–0.5 mm in length. They were, however, often contaminated with very small crystals of another phase, which according to powder photographs was $Ce_6O_4(OH)_4(SO_4)_6$, isomorphous with $U_6O_4(OH)_4(SO_4)_6$ (LUNDGREN 1953). A determination of the crystal structure of this compound is planned.

The crystals of $CeOSO_4H_2O$ are very slightly soluble in water or dilute acids and can be dissolved completely only after evaporation to dryness several times with concentrated nitric acid.

Analysis

A sample, dried in air, was decomposed by fusion with sodium and potassium carbonates. After cooling the melt was leached with water, and the residue, which contained all the cerium, was filtered away. In the filtrate the amount of *sulphate* was determined gravimetrically as barium sulphate.

For the determination of *cerium* a sample was ignited and weighed as CeO_2.

The *water* was determined by HARTWIG–BENDIG's (1941) modification of PENFIELD's method as described by KOLTHOFF and SANDELL (1943).

The *density* of the crystals was determined from the loss of weight in benzene.

	Found		Calculated for $CeOSO_4H_2O$
% SO_3	30.1	30.3	29.63
% CeO_2	64.2	63.6	63.70
% H_2O	6.9	6.6	6.67
Density	4.2_0	4.2_1	4.18

Unit cell and space group

From rotation (around [001] and [010]) and Weissenberg photographs ($hk0$, $hk1$, $hk2$, $h0l$, $h1l$, and $h2l$), taken with Cu K radiation, it was concluded that the crystals are orthorhombic with $a \approx 11.9$ Å, $b \approx 8.2$ Å, and $c \approx 4.3$ Å. More accurate values of the unit cell dimensions were calculated from powder photographs in focusing cameras of the Phragmén type, *cf* Table 1. (Cr K radiation. $\lambda_{\mathrm{Cr}\,K\alpha} = 2.2909$ Å.)

$$a = (11.98_3 \pm 0.01) \text{ Å}$$
$$b = (8.26_7 \pm 0.01) \text{ Å}$$
$$c = (4.33_2 \pm 0.01) \text{ Å}$$
$$V = 429.1 \text{ Å}^3$$

The mean value of the density is 4.2_1 so that there must be $4.0_3 \approx 4$ formula units in the unit cell.

The only reflections systematically absent are:

$h00$ with h odd
$0k0$ with k odd
$00l$ with l odd,

which is characteristic of the space group $D_2^4 - P\,2_1 2_1 2_1$.

Positions of the cerium atoms

To locate the metal atoms in the unit cell the following Patterson projections were calculated (Fig. 1 and 2):

$$P_1 = P\,(u\,v\,p) = \sum_h \sum_k F^2_{hk0} \cos 2\pi\,(hu + kv)$$

$$P_2 = P\,(u\,p\,w) = \sum_h \sum_l F^2_{h0l} \cos 2\pi\,(hu + lw),$$

giving the interatomic distances (u, v, w) projected on the uv plane (P_1) and on the uw plane (P_2). To reduce the absorption effects as much as possible in the calculations, the intensities of the reflections were estimated mainly from the Weissenberg photographs of a very small crystal (about 0.1 mm in length). The weak reflections that were not visible there were estimated from the corresponding photographs of a larger crystal (about 0.5 mm in length). From the estimated intensities the values of F^2 were calculated in the usual way by division with the expression

$$\frac{1 + \cos^2 2\theta}{\sin 2\theta}.$$

Table 1

Powder photographs of $CeOSO_4H_2O$

Cr *K* radiation $\lambda_{Cr\ K\alpha} = 2.2909$ Å

h k l	$10^4 \sin^2 \theta$ calc	$10^4 \sin^2 \theta$ obs	pF^2 calc	*I* obs
1 1 1	983	978	122	st
3 1 0	1014	–	1	–
2 0 1	1065	(1060)	31	(w)
2 2 0	1133	1123	4	vw
2 1 1	1257	(1252)	68	(m)
4 0 0	1462	1463	1	st
0 2 1	1467		105	
3 0 1	1522	(1521)	125	(st)
1 2 1	1559	1556	40	w
3 2 0	1590	1591	120	st
4 1 0	1654	1651	80	m
3 1 1	1714	1706	17	vw
1 3 0	1819	1820	93	m +
2 2 1	1833		14	
2 3 0	2093	2090	34	w
4 0 1	2161	–	2	–
4 2 0	2230	–	0	–
3 2 1	2290	2290	12	vvw
4 1 1	2353	2358	68	w
0 3 1	2427	–	1	–
5 1 0	2476	(2478)	5	(vw)
1 3 1	2519	(2526)	26	(w)
3 3 0	2550	(2560)	10	(vw)
2 3 1	2793	2797	84	st
0 0 2	2797		21	
1 0 2	2889	2897	61	m +
4 2 1	2929	2933	8	vvw
5 0 1	2984	2987	61	st
0 1 2	2989		36	
5 2 0	3052	3056	51	m
0 4 0	3072	3074	38	m
1 1 2	3081		26	
2 0 2	3163	3180	31	st
1 4 0	3163		0	
5 1 1	3176		68	
4 3 0	3190		39	
3 3 1	3250	(3250)	44	(m)
6 0 0	3289	3298	51	m
2 1 2	3355	3358	58	m +
2 4 0	3437	3439	45	m
6 1 0	3481	3490	8	vvw
0 2 2	3565	3572	2	vvw
3 0 2	3619	(3629)	14	(vw)
1 2 2	3656	3662	10	vvw
5 2 1	3752	3753	53	m
0 4 1	3771	3772	36	m
3 1 2	3811	(3821)	48	(w)
1 4 1	3863	3865	48	w
4 3 1	3889	3893	116	st
3 4 0	3894		16	
2 2 2	3931	(3937)	104	(st)
6 0 1	3989	3996	22	vw
5 3 0	4012	4015	20	vvw
6 2 0	4057	(4063)	2	(vvw)
2 4 1	4137	(4138)	23	(vw)
6 1 1	4181	4172	12	vvw
4 0 2	4259	4275	3	vvw
3 2 2	4387	4393	49	w +
4 1 2	4451	(4452)	36	(w)
0 3 2	4525	4522	29	vw
4 4 0	4534		0	
3 4 1	4593	4597	102	m
1 3 2	4616	4612	47	w
7 1 0	4669	4680	34	vw
5 3 1	4711	4715	36	vw
6 2 1	4757	4766	137	m
2 3 2	4891	4895	93	m
1 5 0	4891		13	
6 3 0	5017	5029	3	w +
4 2 2	5027		79	
5 0 2	5081	5088	36	vw
2 5 0	5165	5163	55	w
7 0 1	5176		0	
4 4 1	5233	5238	36	vw
7 2 0	5245		0	
5 1 2	5273	5277	63	vw
3 3 2	5347	5347	50	vw
5 4 0	5356	5362	7	w +
7 1 1	5368		116	
0 5 1	5499	(5500)	13	(vvw)
1 5 1	5590	5584	40	vw
3 5 0	5622	–	2	–
6 3 1	5717	5717	26	vvw
8 0 0	5848	5856	27	w
5 2 2	5849		34	
2 5 1	5865		26	
0 4 2	5869		13	
7 2 1	5944	5958	17	w
1 4 2	5960		92	
4 3 2	5987	5983	24	vw
8 1 0	6040	–	1	–
5 4 1	6055	6055	58	w –
6 0 2	6086	6084	18	vvw
7 3 0	6205	6201	58	vw
2 4 2	6234	6230	37	vw
4 5 0	6262	6255	34	vvw
6 1 2	6278	6279	32	vvw
3 5 1	6321	6316	20	vvw
6 4 0	6361	6369	39	vw –
1 0 3	6385	–	0	–
0 1 3	6486	6482	29	vvw
8 0 1	6547	6545	11	vvw

Table 1 (cont.)

$h\,k\,l$	$10^4 \sin^2\theta$ calc	$10^4 \sin^2\theta$ obs	pF^2 calc	I obs	$h\,k\,l$	$10^4 \sin^2\theta$ calc	$10^4 \sin^2\theta$ obs	pF^2 calc	I obs
1 1 3	6577	6574	14	vvw	2 1 3	6851	6849	32	w +
8 2 0	6616	—	0	—	6 2 2	6854		33	
2 0 3	6659	6649	36	vw	7 3 1	6904	6909	32	vvw
3 4 2	6691	(6686)	30	(vw)	0 6 0	6912		8	
8 1 1	6739	6737	32	vw −	4 5 1	6961	—	10	—
5 3 2	6809	—	7	—					

The horizontal line marks the boundary between the angular ranges of the two different focusing cameras used.

Reflections systematically absent in space group D_2^4 have been omitted. Reflections coinciding with β reflections are given in brackets.

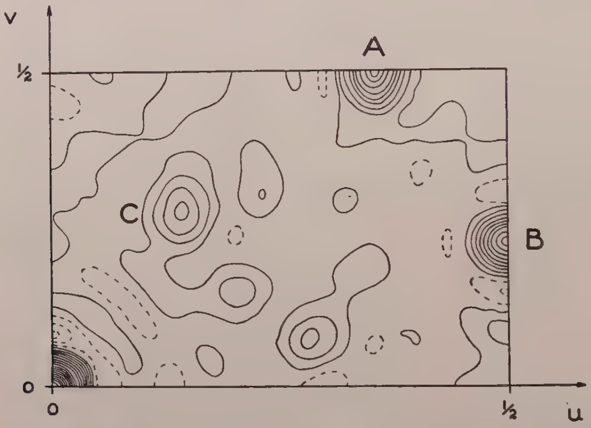


Fig. 1. Patterson analysis of $CeOSO_4H_2O$. The function $P_1 = P(uvp) = \sum_h \sum_k F^2_{h\,k\,0}\, 2\pi\,(hu + kv)$. Dotted lines indicate negative values.

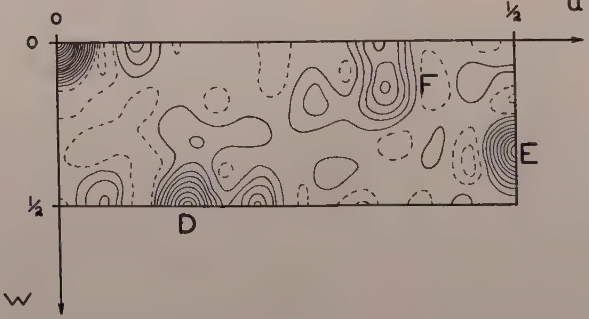


Fig. 2. Patterson analysis of $CeOSO_4H_2O$. The function $P_2 = P(upw) = \sum_h \sum_l F^2_{h\,0\,l} \cos 2\pi\,(hu + lw)$. Dotted lines indicate negative values.

Now, in D_2^4 there is only one point position, namely 4 (a): $x\,y\,z$; $\frac{1}{2} - x, \bar{y}, \frac{1}{2} + z$; $\frac{1}{2} + x, \frac{1}{2} - y, \bar{z}$; $\bar{x}, \frac{1}{2} + y, \frac{1}{2} - z$; in which the cerium atoms should then be situated. The Ce–Ce distances would then be:

$$+\left(\tfrac{1}{2} \pm 2x, 2y, \tfrac{1}{2}\right)$$
$$\pm\left(\tfrac{1}{2}, \tfrac{1}{2} \pm 2y, 2z\right)$$
$$\pm\left(2x, \tfrac{1}{2}, \tfrac{1}{2} \pm 2z\right)$$

The Patterson projections on the uv and uw planes would thus show the following maxima

in the uv plane:			in the uw plane:		
I.	$\pm(2x, \frac{1}{2})$	weight 2	IV.	$\pm(\frac{1}{2} - 2x, \frac{1}{2})$	weight 2
II.	$\pm(\frac{1}{2}, \frac{1}{2} - 2y)$	» 2	V.	$\pm(\frac{1}{2}, 2z)$	» 2
III.	$\pm(\frac{1}{2} \pm 2x, 2y)$	» 1	VI.	$\pm(2x, \frac{1}{2} \pm 2z)$	» 1

If we identify I and II with the high peaks A and B in Fig. 1 and V with the high peak E in Fig. 2, we get the following values for the parameters of the cerium atoms (we need only consider the range $0 \le x \le \frac{1}{4}$, $0 \le y \le \frac{1}{4}$, $0 \le z \le \frac{1}{4}$):

$$x_{\text{Ce}} \approx 0.179 \quad y_{\text{Ce}} \approx 0.136 \quad z_{\text{Ce}} \approx 0.171$$

Using these parameters also the distances III, IV, and VI above were calculated. They were then found to correspond to the maxima C in Fig. 1 and D and F in Fig. 2. The heights of the peaks also corresponded to the calculated weights. We thus conclude:

$$4\ \text{Ce in } \mathrm{D}_2^4\ 4(a) \text{ with } x \approx 0.179 \quad y \approx 0.136 \quad z \approx 0.171.$$

Positions of the light atoms

Electron density projections

In addition to the Ce–Ce peaks the Patterson projections in Fig. 1 and 2 show some small maxima, which could be ascribed to the Ce–S and perhaps also to some Ce–O distances. From these small maxima it would be possible to find out at least the positions of the sulphur atoms in the unit cell. It would, however, be more favourable to determine their sites from a map giving the electron density, as such a map gives maxima that are sharper and less in number than those of the corresponding vector map.

For $\mathrm{CeOSO_4H_2O}$ electron density projections on the xy, xz, or yz planes could rather easily be calculated, as in D_2^4 the projections of the atomic arrangement on these planes have centres of symmetry, even though the spatial arrangement has not. Further, the signs of most of the F-values in $hk0$, $h0l$, or $0kl$ could be determined from the contributions given by the cerium atoms, because of their large reflecting power compared to those of the sulphur and oxygen atoms.

Now, as the c axis is very short, the xy projection of the electron density would show very little overlapping, and it would consequently be easier to locate the light atoms in this projection than in any of the others. Thus $\varrho(xyp)$ was calculated, at first using only those F-values for which the signs could be determined from the cerium contributions, and then completing it with the few remaining ones the signs of which were determined by the sulphur and oxygen atoms.

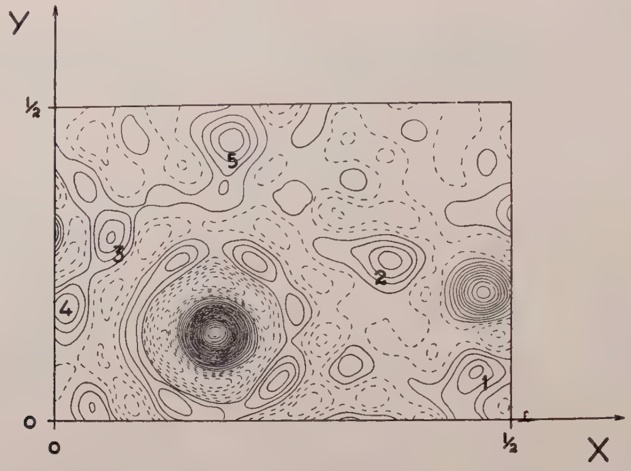


Fig. 3. Electron density of $CeOSO_4H_2O$ projected on the xy plane. Dotted lines indicate negative values. In the cerium peak only every second contour has been marked out. The figures 1–5 denote the oxygen maxima.

Fig. 3 shows the electron density projected on the xy plane. It has two very pronounced maxima, the highest at $x = 0.178$ $y = 0.137$ giving the position of the cerium atom in this quadrant, and one, lower, at $x = 0.470$ $y = 0.202$ giving the position of the sulphur atom. In addition there are some small peaks at

1. $x = 0.465$ $y = 0.074$
2. $x = 0.371$ $y = 0.251$
3. $x = 0.064$ $y = 0.296$
4. $x = 0.013$ $y = 0.180$
5. $x = 0.197$ $y = 0.444$

These small maxima have all nearly the same heights, which are about $\frac{1}{12}$ of that of cerium and $\frac{1}{3}$ of sulphur. It would thus seem probable that they correspond to five of the six oxygen positions in the unit cell. However, the peaks might be a little displaced from the correct oxygen positions by diffraction effects caused by the heavy cerium atom. (That such effects occur is seen from the ring surrounding the cerium atom in Fig. 3.) More reliable coordinates for the oxygen atoms would then probably be obtained, if the cerium atom were subtracted from the Fourier image in Fig. 3. For that reason a new electron density projection was calculated from the expression

$$\varrho_1(xyp) = \sum_h \sum_k \left(|F_{\mathrm{obs}\,(hk0)}| - |F_{\mathrm{Ce}\,(hk0)}|\right) \cos 2\pi\left[(hx+ky) - \alpha_{hk0}\right],$$

where $F_{\mathrm{obs}\,(hk0)}$ are the observed structure factors normalized by multiplication with the constant $\dfrac{\sum_{h,k} |F_{\mathrm{calc}\,(hk0)}|}{\sum_{h,k} |F_{\mathrm{obs}\,(hk0)}|}$. (The temperature factor, calculated with the ordinary procedure, was found to be negligible. Probably it roughly cancels out with the absorption effect.) $F_{\mathrm{Ce}\,(hk0)}$ are the calculated cerium contributions to the structure factors. The phase angles α_{hk0} are multiples of $\frac{1}{2}\pi$.

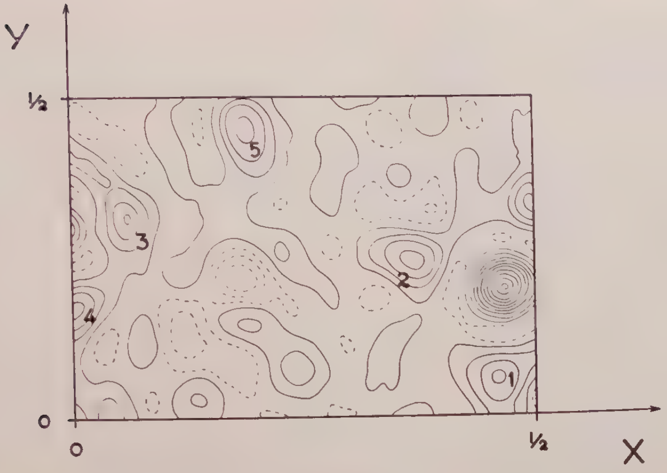


Fig. 4. Electron density of $CeOSO_4H_2O$ projected on the xy plane and with the cerium atom subtracted. Dotted lines indicate negative values. The figures 1–5 denote the oxygen maxima.

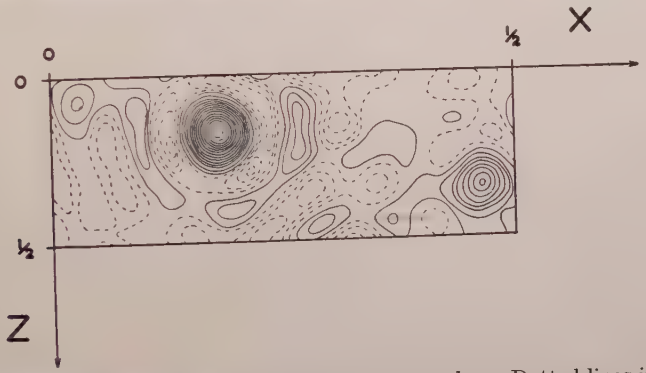


Fig. 5. Electron density of $CeOSO_4H_2O$ projected on the xz plane. Dotted lines indicate negative values. In the cerium peak only every second contour has been marked out.

Fig. 4 shows $\varrho_1(xyp)$. As is seen the cerium atom and most of its diffraction effects have disappeared, but the maxima corresponding to the sulphur and the five oxygen atoms are still of the same heights. The position of the sulphur peak is the same as in Fig. 3, but, as was expected, the positions of the oxygen maxima have changed a little. Their new coordinates, probably in better agreement with the correct oxygen parameters, are:

1. $x = 0.459$ $y = 0.058$
2. $x = 0.368$ $y = 0.246$
3. $x = 0.060$ $y = 0.311$
4. $x = 0.001$ $y = 0.173$
5. $x = 0.189$ $y = 0.452$

To find out the z parameters of the light atoms, the electron density projection on the xz plane, $\varrho(xpz)$, was calculated (Fig. 5). Here the cerium and sulphur atoms as before stand out very clearly, the cerium at $x = 0.180$ $z = 0.171$ and the sulphur at $x = 0.464$ $z = 0.347$. The x parameter of the cerium atom is within the limits the same as that obtained from $\varrho(xyp)$, but the x parameter of the sulphur atom has changed a little. The value obtained from $\varrho(xyp)$ should, however, be more correct, since in the calculation of this projection more F-values were used.

In contrast to $\varrho(xyp)$ there are no separate maxima in $\varrho(xpz)$ that could be ascribed to oxygen atoms. This was also to be expected, as the b axis is relatively long, 8.27 Å, and much overlapping should occur. The z parameters of the oxygen atoms must then be determined in some other way.

Thus, from the electron density projections the following parameters could be obtained:

4 Ce in D_2^4 4(a) with $x = 0.178$ $y = 0.137$ $z = 0.171$
4 S » » » » $x = 0.470$ $y = 0.202$ $z = 0.34_7$
4 O$_1$ » » » » $x = 0.45_9$ $y = 0.05_8$
4 O$_2$ » » » » $x = 0.36_8$ $y = 0.24_6$
4 O$_3$ » » » » $x = 0.06_0$ $y = 0.31_1$
4 O$_4$ » » » » $x = 0.00_1$ $y = 0.17_3$
4 O$_5$ » » » » $x = 0.18_9$ $y = 0.45_2$

4(a): xyz; $\frac{1}{2} - x, \bar{y}, \frac{1}{2} + z$; $\frac{1}{2} + x, \frac{1}{2} - y, \bar{z}$; $\bar{x}, \frac{1}{2} + y, \frac{1}{2} - z$.

Geometrical considerations

Of the five oxygen atoms, the positions of which have been found from the electron density projections, O$_1$, O$_2$, O$_3$, and O$_4$ are situated close to sulphur atoms and surely belong to the sulphate groups in the unit cell. Assuming that these have approximately the same dimensions as in previously described structures (*i.e.* a regular tetrahedron with O–O $= 2.4_0$ Å and S–O $= 1.4_7$ Å, *cf.* WELLS 1949), it is then possible to calculate the z parameters of the atoms O$_1$–O$_4$. During this calculation, however, it was found that O$_4$ probably must be shifted a little in the xy plane, as otherwise at least one distance between O$_4$ and one of the atoms O$_1$, O$_2$, or O$_3$ was found to be too small, if the other O–O distances within the sulphate groups were assumed to be 2.35–2.45 Å. Now, in Fig. 4 the peak corresponding to O$_4$ is somewhat deformed. It is then possible that the top of this peak does not correspond to the correct parameters of O$_4$. In fact, if y_{O_4} is diminished with 0.1 Å, *i.e.* if $y_{O_4} = 0.16_1$, all the O–O distances within a sulphate group can be in the normal range. The new position is still within the highest contour at the O$_4$ peak in Fig. 4. These two facts suggest that $x_{O_4} = 0.00_1$ $y_{O_4} = 0.16_1$ are closer to the correct parameters than $x_{O_4} = 0.00_1$ $y_{O_4} = 0.17_3$ as given above.

From the dimensions of the sulphate group the following two sets of z parameters for the oxygen atoms O$_1$–O$_4$ were found possible:

A.	B.
$z_{O_1} = 0.52$	$z_{O_1} = 0.17$
$z_{O_2} = 0.18$	$z_{O_2} = 0.51$
$z_{O_3} = 0.88$	$z_{O_3} = 0.42$
$z_{O_4} = 0.43$	$z_{O_4} = 0.87$

A gives $z_{O_1} > z_S$ and B gives $z_{O_1} < z_S$. To see which of these two possibilities is more likely we calculate for each of the oxygen atoms the distance to its nearest cerium atom. The following values are then obtained:

A.	B.
$Ce-O_1 = 2.3_9$ Å	$Ce-O_1 = 3.0_8$ Å
$Ce-O_2 = 2.4_5$ Å	$Ce-O_2 = 2.8_7$ Å
$Ce-O_3 = 2.3_8$ Å	$Ce-O_3 = 2.2_9$ Å
$Ce-O_4 = 2.4_1$ Å	$Ce-O_4 = 2.5_0$ Å

In CeO_2 the shortest distances Ce O are 2.34 Å (GOLDSCHMIDT and THOMASSEN 1923, MAGNÉLI and KIHLBORG 1951), and also in the structure of $CeOSO_4H_2O$ we should expect that they are about the same. We see then that in A all four of the sulphate oxygen atoms are in contact with cerium, but in B only two of them are situated close to cerium. It seems then probable that A corresponds to the real arrangement, and we conclude that O_1–O_4 should be situated in the following way:

4 O_1 in D_2^4 4(a) with $x = 0.45_9$ $y = 0.05_8$ $z = 0.52$
4 O_2 » » » » $x = 0.36_8$ $y = 0.24_6$ $z = 0.18$
4 O_3 » » » » $x = 0.06_0$ $y = 0.31_1$ $z = 0.88$
4 O_4 » » » » $x = 0.00_1$ $y = 0.16_1$ $z = 0.43$

Now, when the positions of the atoms O_1–O_4 have been determined, we try to find out the z paramter of O_5. This atom is situated at a rather large distance from the sulphate groups, and if the minimum permissible distance between oxygen atoms not belonging to the same sulphate group is assumed to be 2.60 Å, we find that the position of O_5 is not influenced at all by the atoms O_1, O_2, and O_4. The xy projection of the distance O_3–O_5, however, is only 1.9_3 Å, and as a consequence its projection on the z axis should be at least 1.7_5 Å. The z parameter of O_5 should then satisfy the condition $0.29 \leq z_{O_5} \leq 0.48$. The shortest distance Ce–O_5 is, with z_{O_5} between these limits, $\geq 2.6_6$ Å, the equality sign being valid when $z_{O_5} = 0.29$. As we might expect the distance Ce–O_5 to be as close to 2.34 Å as possible (*cf.* above), we shall assume preliminarily that the parameters of O_5 are:

4 O_5 in D_2^4 4(a) with $x = 0.18_9$ $y = 0.45_2$ $z = 0.29$

In the unit cell there are still four oxygen atoms (O_6) the positions of which have to be determined. As nothing is seen in $\varrho(xyp)$, $\varrho_1(xyp)$, or $\varrho(xpz)$ that could give any hints as to their sites, their parameters have to be determined altogether from space considerations. We may then observe that, because of the short c axis, the xy projections of all O–O_6 and Ce–O_6 distances should be $\geq \sqrt{2.60^2 - \frac{c^2}{4}} = 1.44$ Å and $\geq \sqrt{2.34^2 - \frac{c^2}{4}} = 0.89$ Å, if the interatomic distances are as above assumed to be O–O ≥ 2.60 Å, and Ce–O ≥ 2.34 Å. Nor should any oxygen atom O_6 be situated closer to the 2_1 axes $\pm \frac{1}{4} 0 z$ and $\pm \frac{1}{4} \frac{1}{2} z$ than 0.72 Å, as otherwise some O_6–O_6 distances would be too short. The following cylinders, parallel to the c axis, thus constitute domains forbidden for the oxygen atoms O_6 (irrespective of the z parameters of the atoms already located):

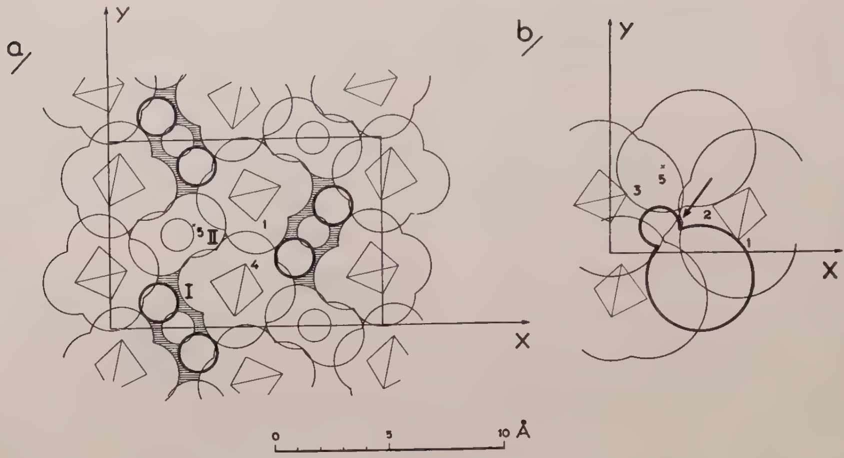


Fig. 6. a) Survey over the space free (shaded areas) for the oxygen atoms O_6. The circles enclose domains forbidden for the centres of O_6 because of the following conditions:
Ce–O_6 (projected on the xy plane) ≥ 0.89 Å (thick circles)
O–O_6 (» » » » ») ≥ 1.44 Å (large thin circles).
distance from a 2_1 axis to the centre of an O_6 atom ≥ 0.72 Å (small thin circles).
As for the domains I and II see the text page 68.

b) Section at $z = 0.67$ through the domain I in a). The circles enclose domains forbidden for the centres of the oxygen atoms O_6 because of the conditions Ce $-$ $O_6 \geq 2.34$ Å (thick circles) and O–$O_6 \geq 2.60$ Å (thin circles). The arrow points out the very small area left free for an O_6 centre.

In a) and b) those oxygen atoms O_1–O_5 that are mentioned in the text have been marked out; O_1–O_4 belong to sulphate groups, O_5 to a water molecule.

a. Cylinders with radii 1.44 Å and with axes going through the centres of the oxygen atoms O_1–O_5.
b. Cylinders with radii 0.89 Å and with axes going through the centres of the cerium atoms.
c. Cylinders with radii 0.72 Å and with axes $\pm \frac{1}{4} 0 z$ and $\pm \frac{1}{4} \frac{1}{2} z$.

To get a survey over the space forbidden for O_6 irrespective of the z parameters of the atoms already located, an xy projection of the unit cell was drawn (Fig. 6 a) with all these cylinders marked out. As is seen, even these rather wide conditions restrict the x and y parameters of O_6 to two domains, marked out as I and II in the figure. Now, the area of II is very small, and if O_6 would have x and y coordinates corresponding to it, it should be necessary that $\frac{1}{2} - z_{O_1}$, $\bar{z}_{O_4}$, and $z_{O_5} - \frac{1}{2}$ are nearly the same. As this is not the case, we conclude that the oxygen atoms O_6 should be situated somewhere in I and areas equivalent to it.

To determine the positions of O_6 more exactly a set of sections at different z values were drawn through the domain I in Fig. 6 a, and in each section the areas forbidden for O_6 because of the conditions Ce–$O_6 \geq 2.34$ Å and O–$O_6 \geq 2.60$ Å were marked out. It was then found that only at $z = 0.67$ there is a very small area left free. This is shown in Fig. 6 b, where the thick circles enclose domains forbidden for O_6 because of the first condition and the thin circles domains forbidden because of the second. Only the very small area at the arrow-head close to the cerium atoms is left free. This position seems also to be very probable for a non-sulphate oxygen atom, as there it would be in contact with three cerium atoms. We thus conclude:

$$4\ \mathrm{O}_6 \text{ in } \mathrm{D}_2^4\ 4(\mathrm{a}) \text{ with } x = 0.25_3\ y = 0.12_9\ z = 0.67$$

(In the electron density projections this position is situated in the diffraction ring surrounding the cerium atom. It is then natural that the positions of O_6 could not be obtained from these maps.)

As is seen from Fig. 6 b, the area free for O_6 is determined by the contacts Ce–O_6, O_1–O_6, O_2–O_6, and O_3 O_6, whereas O_5 has no influence (the xy projection of the distance O_5–O_6 is 2.7_8 Å as calculated from the parameters given above). On the other hand, O_6 does not influence the position of O_5, and thus there seems to be no reason to change the preliminary parameters obtained for O_5. However, we may observe that the accuracy of z_{O_5} is much lower than that of the other oxygen atoms.

In the unit cell there are also eight hydrogen atoms, which should be situated close to some of the oxygen atoms, probably forming hydroxyl bonds. They could then be distributed over the sulphate groups or the non-sulphate oxygen atoms O_5 and O_6, forming:

A.	B.	C.	D.
4 H_2SO_4	4 HSO_4^-	4 SO_4^{2-}	4 SO_4^{2-}
8 O^{2-}	4 OH^-	8 OH^-	4 H_2O
	4 O^{2-}		4 O^{2-}

A or B does not seem very probable, as in these cases there would be H_2SO_4 molecules (or HSO_4 ions) close to O^{2-} ions. As the proton affinity is much greater for O^{2-} than for HSO_4 (or SO_4^{2-}), the arrangement of hydrogen atoms would surely change from A or B to C or D. It seems then probable that the hydrogen atoms are attached to the oxygen atoms O_5 or O_6 or distributed over both these positions. Now, each atom O_5 is in contact with only one cerium atom (at the rather long distance 2.6_6 Å), whereas each atom O_6 is in close contact (2.3_4 Å) with three cerium atoms. Because of the high charge on the cerium ions it seems then likely that all the hydrogen atoms are attached to O_5 rather than evenly distributed over O_5 and O_6. The case D above then seems to be more likely than C.

Final parameters of $CeOSO_4H_2O$

The following structure is thus proposed for $CeOSO_4H_2O$:
Space group: $D_2^4 - P\,2_12_12_1$. 4 formula units per unit cell.

	x	y	z
4 Ce in 4(a)	0.178 ± 0.002	0.137 ± 0.002	0.171 ± 0.004
4 S in 4(a)	0.470 ± 0.005	0.202 ± 0.005	$0.34_7 \pm 0.01$
4 O_1 in 4(a)	0.45_9	0.05_8	0.52
4 O_2 in 4(a)	0.36_8	0.24_6	0.18
4 O_3 in 4(a)	0.06_0	0.31_1	0.88
4 O_4 in 4(a)	0.00_1	0.16_1	0.43
4 $O_5(H_2O)$ in 4(a)	0.18_9	0.45_2	0.29
4 O_6 in 4(a)	0.25_3	0.12_9	0.67

4(a): xyz; $\frac{1}{2} - x, \bar{y}, \frac{1}{2} + z$; $\frac{1}{2} + x, \frac{1}{2} - y, \bar{z}$; $\bar{x}, \frac{1}{2} + y, \frac{1}{2} - z$.

Table 2

Comparison between calculated and observed structure factors from WEISSENBERG photograph of $CeOSO_4H_2O$. Cu K radiation.

$h\,k\,l$	F calc	F obs
2 0 0	− 15	15
4 0 0	+ 7	8
6 0 0	+ 45	44
8 0 0	− 33	34
10 0 0	0	< 7
12 0 0	+ 16	13
14 0 0	− 33	28
1 1 0	+ 32	25
2 1 0	− 24	24
3 1 0	− 5	< 6
4 1 0	+ 40	41
5 1 0	− 10	9
6 1 0	− 13	13
7 1 0	+ 26	29
8 1 0	− 4	< 6
9 1 0	− 5	< 6
10 1 0	+ 37	34
11 1 0	− 4	10
12 1 0	− 12	14
13 1 0	+ 19	17
14 1 0	+ 2	< 5
15 1 0	− 14	14
0 2 0	− 23	15
1 2 0	+ 12	8
2 2 0	− 9	10
3 2 0	− 49	41
4 2 0	+ 1	< 4
5 2 0	+ 32	31
6 2 0	− 7	7
7 2 0	+ 2	< 6
8 2 0	− 1	< 6
9 2 0	− 20	18
10 2 0	+ 3	7
11 2 0	+ 36	34
12 2 0	0	< 6
13 2 0	− 5	< 6
14 2 0	+ 3	< 5
15 2 0	− 9	8
1 3 0	− 43	34
2 3 0	− 26	27
3 3 0	+ 14	12
4 3 0	+ 28	31
5 3 0	+ 20	23
6 3 0	− 8	12
7 3 0	− 34	33
8 3 0	− 12	11
9 3 0	+ 11	17
10 3 0	+ 12	14
11 3 0	+ 1	< 7
12 3 0	− 17	17
13 3 0	− 29	26
14 3 0	− 1	<4
0 4 0	− 39	32
1 4 0	− 2	< 5
2 4 0	+ 30	30
3 4 0	+ 18	16
4 4 0	+ 1	< 5
5 4 0	− 12	12
6 4 0	− 28	33
7 4 0	− 1	< 6
8 4 0	+ 25	34
9 4 0	+ 5	7
10 4 0	− 7	12
11 4 0	− 15	20
12 4 0	− 24	26
13 4 0	+ 1	< 5
14 4 0	+ 24	19
1 5 0	− 16	14
2 5 0	+ 33	35
3 5 0	+ 7	10
4 5 0	− 26	30
5 5 0	+ 10	14
6 5 0	+ 12	15
7 5 0	− 7	7
8 5 0	+ 17	21
9 5 0	+ 13	21
10 5 0	− 23	29
11 5 0	+ 11	15
12 5 0	+ 16	23
0 6 0	+ 18	14
1 6 0	− 30	28
2 6 0	− 5	<6
3 6 0	+ 23	29
4 6 0	+ 2	< 6
5 6 0	− 30	34
6 6 0	+ 14	18
7 6 0	+ 5	7
8 6 0	− 12	17
9 6 0	+ 25	29
10 6 0	+ 4	< 6
11 6 0	− 21	24
12 6 0	+ 11	9
1 7 0	+ 31	30
2 7 0	+ 5	< 7
3 7 0	− 13	14
4 7 0	− 3	< 7
5 7 0	− 23	30
6 7 0	+ 6	12
7 7 0	+ 21	29
8 7 0	+ 6	< 7
9 7 0	− 22	28
10 7 0	− 4	< 5
11 7 0	− 13	10
0 8 0	+ 19	21
1 8 0	+ 15	17
2 8 0	− 25	26
3 8 0	− 10	13
4 8 0	− 12	18
5 8 0	+ 16	20
6 8 0	+ 18	23
7 8 0	+ 4	5
8 8 0	− 13	18
9 8 0	− 12	10
1 9 0	+ 7	< 7
2 9 0	− 22	25
3 9 0	− 2	< 6
4 9 0	+ 25	25
5 9 0	− 1	< 5
6 9 0	− 17	20
7 9 0	+ 4	< 5
8 9 0	− 17	14
0 10 0	− 19	19
1 10 0	+ 8	10
2 10 0	+ 16	18
3 10 0	− 19	20
4 10 0	+ 7	9
5 10 0	+ 14	12
1 0 1	+ 8	6
2 0 1	+ 25	28
3 0 1	− 50	49
4 0 1	− 7	7
5 0 1	+ 35	31
6 0 1	+ 21	22
7 0 1	− 3	< 5
8 0 1	+ 15	18
9 0 1	− 22	27
10 0 1	− 11	14
11 0 1	+ 35	39
12 0 1	+ 11	17
13 0 1	− 7	14
14 0 1	+ 2	< 5
15 0 1	− 9	6
0 1 1	+ 9	11
1 1 1	35	31
2 1 1	26	20
3 1 1	13	9
4 1 1	26	22
5 1 1	26	21

Table 2 (cont.)

h k l	*F* calc	*F* obs	*h k l*	*F* calc	*F* obs	*h k l*	*F* calc	*F* obs
6 1 1	11	9	0 5 1	− 16	18	5 9 1	21	17
7 1 1	34	31	1 5 1	20	19	6 9 1	14	13
8 1 1	18	15	2 5 1	16	17	7 9 1	22	13
9 1 1	13	14	3 5 1	14	15			
10 1 1	18	21	4 5 1	10	9	0 10 1	− 16	14
11 1 1	8	11	5 5 1	17	18	1 10 1	15	13
12 1 1	11	15	6 5 1	15	15	2 10 1	10	11
13 1 1	20	23	7 5 1	27	27	3 10 1	11	9
14 1 1	6	7	8 5 1	21	24			
			9 5 1	17	21	0 0 2	− 29	28
0 2 1	− 45	35	10 5 1	4	< 6	1 0 2	− 35	31
1 2 1	20	17	11 5 1	9	11	2 0 2	+ 25	22
2 2 1	12	9	12 5 1	13	12	3 0 2	+ 17	17
3 2 1	11	10	13 5 1	20	10	4 0 2	+ 8	5
4 2 1	9	7				5 0 2	+ 27	24
5 2 1	23	17	0 6 1	+ 21	20	6 0 2	− 19	18
6 2 1	37	30	1 6 1	14	15	7 0 2	− 17	17
7 2 1	13	15	2 6 1	24	17	8 0 2	+ 16	17
8 2 1	29	26	3 6 1	16	14	9 0 2	+ 17	20
9 2 1	15	17	4 6 1	13	15	10 0 2	− 2	< 7
10 2 1	5	<7	5 6 1	10	13	11 0 2	+ 13	13
11 2 1	9	9	6 6 1	26	26	12 0 2	− 9	11
12 2 1	14	14	7 6 1	20	24	13 0 2	− 18	19
13 2 1	12	15	8 6 1	24	25	14 0 2	+ 18	17
14 2 1	23	20	9 6 1	7	8			
			10 6 1	12	11	1 0 3	− 1	< 5
0 3 1	+ 4	6	11 6 1	10	13	2 0 3	− 27	26
1 3 1	16	11				3 0 3	+ 5	< 6
2 3 1	29	23	0 7 1	− 8	13	4 0 3	+ 27	24
3 3 1	21	19	1 7 1	5	9	5 0 3	− 2	< 7
4 3 1	34	29	2 7 1	25	26	6 0 3	− 21	20
5 3 1	19	19	3 7 1	20	25	7 0 3	+ 5	< 7
6 3 1	16	16	4 7 1	25	28	8 0 3	− 18	19
7 3 1	18	17	5 7 1	10	15	9 0 3	+ 6	< 7
8 3 1	11	11	6 7 1	17	23	10 0 3	+ 24	29
9 3 1	21	20	7 7 1	4	6	11 0 3	− 1	< 6
10 3 1	28	30	8 7 1	15	16	12 0 3	− 23	28
11 3 1	11	15	9 7 1	11	10			
12 3 1	14	16	10 7 1	19	16	0 0 4	− 18	21
13 3 1	13	13				1 0 4	+ 17	26
14 3 1	8	9	0 8 1	− 9	19	2 0 4	+ 3	< 7
			1 8 1	15	20	3 0 4	− 8	11
0 4 1	+ 27	27	2 8 1	11	22	4 0 4	− 1	< 6
1 4 1	22	19	3 8 1	15	22	5 0 4	− 22	20
2 4 1	15	15	4 8 1	18	21	6 0 4	− 10	9
3 4 1	32	29	5 8 1	18	20	7 0 4	+ 17	19
4 4 1	19	23	6 8 1	11	11	8 0 4	+ 9	11
5 4 1	24	25	7 8 1	10	12	9 0 4	− 15	18
6 4 1	15	12	8 8 1	11	6			
7 4 1	5	< 6	9 8 1	17	13	1 0 5	+ 1	< 5
8 4 1	13	15				2 0 5	+ 10	7
9 4 1	19	26	0 9 1	+ 14	18	3 0 5	+ 28	25
10 4 1	13	19	1 9 1	22	25	4 0 5	− 15	13
11 4 1	21	25	2 9 1	5	11	5 0 5	− 11	15
12 4 1	8	13	3 9 1	5	9	6 0 5	+ 8	11
13 4 1	10	13	4 9 1	7	9			
						0 1 2	+ 27	28

Table 2 (cont.)

h k l	*F* calc	*F* obs	*h k l*	*F* calc	*F* obs	*h k l*	*F* calc	*F* obs
1 1 2	16	16	1 1 3	12	15	3 1 4	13	15
2 1 2	24	27	2 1 3	18	19	4 1 4	9	13
3 1 2	22	20	3 1 3	24	21	5 1 4	21	18
4 1 2	19	20	4 1 3	15	15	6 1 4	16	15
5 1 2	25	23	5 1 3	20	18	7 1 4	4	7
6 1 2	18	18	6 1 3	22	20	8 1 4	14	16
7 1 2	8	8	7 1 3	2	< 5	9 1 4	16	14
8 1 2	19	16	8 1 3	21	19	10 1 4	14	14
9 1 2	18	17	9 1 3	13	16	0 1 5	+ 11	10
10 1 2	16	17	10 1 3	6	7	1 1 5	17	14
11 1 2	16	18	11 1 3	17	20	2 1 5	13	12
12 1 2	14	16	12 1 3	14	17	3 1 5	11	9
13 1 2	14	18	0 1 4	− 21	19	4 1 5	17	12
14 1 2	15	16	1 1 4	18	15	5 1 5	12	9
0 1 3	− 24	26	2 1 4	18	15	6 1 5	14	10

For F_{calc} the sign is given at the reflections having either A or $B=0$.

The accuracy of the oxygen parameters is estimated to be ± 0.01 in x and y for O_1—O_6, and ± 0.03 in z for O_1—O_4 and O_6. z_{O_5} might be a little more uncertain.

Using these parameters the structure factors for $hk0$, $hk1$, $h0l$, and $h1l$ were calculated and compared to the observed structure factors from the corresponding Weissenberg photographs (Table 2). The agreement was then found to be rather good, the reliability index $R = \frac{\sum ||F_{obs}| - |F_{calc}||}{\sum |F_{obs}|}$ being 0.19 for $hk0$ and 0.16 for $h0l$ reflections (absent reflections included). According to BOOTH (1948) R usually has a value between the limits 0.12 and 0.22 for a completed structure. However, for a crystal like this containing both heavy and light atoms a low value of the reliability index should not be taken as a definite proof that the structure as a whole is true, but only that the arrangement proposed for the heavy atoms should be essentially correct.

Description of the structure

Fig. 7 shows the structure of $CeOSO_4H_2O$. The cerium ions form zigzag strings parallel to the c axis. Within these strings they are joined by O^{2-} ions, each of which is in contact with three cerium atoms. This arrangement of the metal and oxygen atoms having the empirical composition $(CeO^{2+})_n$ has been drawn separately in Fig. 8.

The distances from one cerium atom to its four cerium neighbours in the string are 3.58 Å and 4.33 Å. The first distance is very short, much shorter than the Ce − Ce distances in CeO_2 (3.83 Å) (GOLDSCHMIDT and THOMASSEN 1923, MAGNÉLI and KIHLBORG 1951). It is in fact a little less than the interatomic distances in cerium metal, which are 3.63 Å (3.49 Å in the high pressure modification) (HULL 1921, QUILL 1932, KLEMM and BOMMER 1937, LAWSON and TANG 1949). However, in $CeOSO_4H_2O$ there are probably no bonds between the metal ions, as the two oxygen

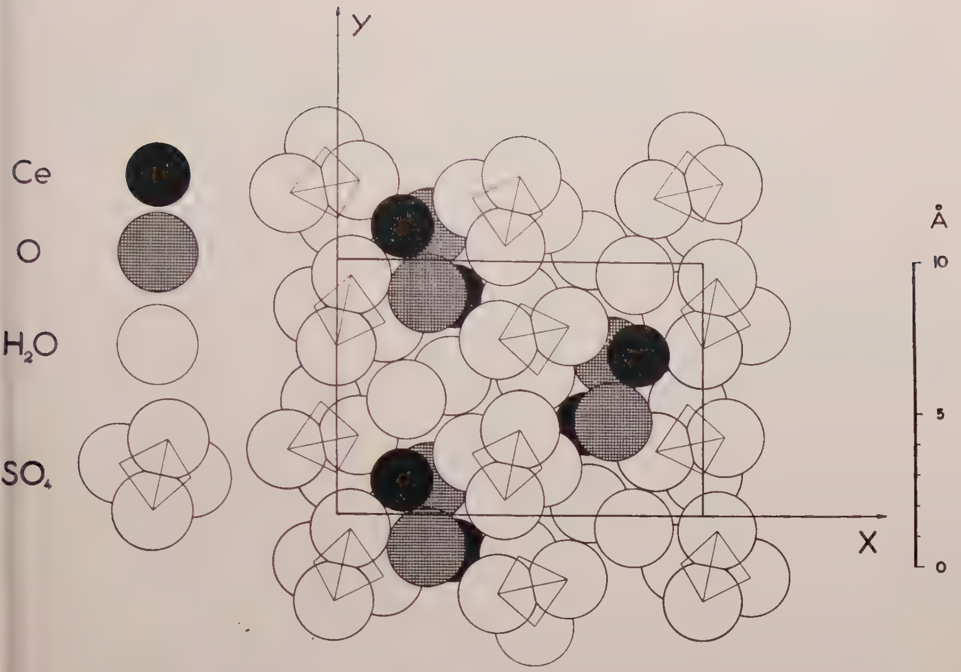
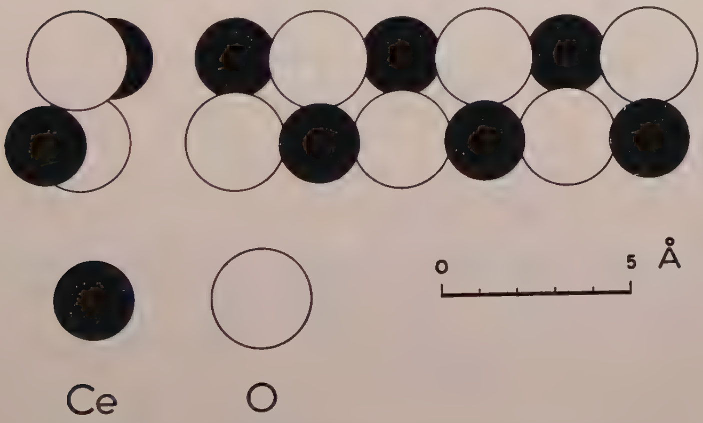


Fig. 7. The structure of $CeOSO_4H_2O$, viewed along [001].



ig. 8. The $(CeO^{2+})_n$ string. The figure shows the arrangement of the Ce^{4+} and O^{2-} ions, viewed along [001] (left) and along [100] (right).

toms separating the two closest cerium ions are only 3.0_4 Å apart, which value s rather near the distance of van der Waal's contact between oxygen atoms. A ond between the cerium atoms should then pass through the outer parts of the xygen ions, which does not seem probable.

The arrangement of the metal and oxygen ions in $\mathrm{CeOSO_4H_2O}$ is different from that found in the corresponding thorium and uranium(IV) compounds (LUNDGREN 1950, 1952). These two salts are isomorphous, and, as in $\mathrm{CeOSO_4H_2O}$, their metal ions are arranged in zigzag strings, but the Me–Me distances are longer, about the same as in the corresponding dioxides. The metal ions are then joined by a double row of $\mathrm{OH^-}$ ions, not by $\mathrm{O^{2-}}$ as in $\mathrm{CeOSO_4H_2O}$, and each $\mathrm{OH^-}$ is in contact with only two metal ions.

In the structure of $\mathrm{CeOSO_4H_2O}$ the $(\mathrm{CeO^{2+}})_n$ strings are connected with sulphate ions, so that every sulphate oxygen atom is in contact with one cerium atom. The water of crystallization is contained in channels in the structure, and each of the water molecules is in contact with a cerium atom. In this way every cerium is surrounded by eight oxygen atoms, a coordination which is expected for ions of this radius ratio. The coordination figure is a distorted square Archimedean antiprism.

The interatomic distances for the structure proposed will be (in Å):

$\mathrm{Ce - O_1} = 2.3_9$
$\mathrm{Ce - O_2} = 2.4_5$
$\mathrm{Ce - O_3} = 2.3_8$
$\mathrm{Ce - O_4} = 2.4_1$
$\mathrm{Ce - O_5} = 2.6_6$
$\mathrm{Ce - 3\ O_6} = 2.3_4\ ^{(*)}$
$\mathrm{S - O_1} = 1.4_2$
$\mathrm{S - O_2} = 1.4_7$
$\mathrm{S - O_3} = 1.4_6$
$\mathrm{S - O_4} = 1.5_3$
$\mathrm{O_1 - O_2} = 2.4_0\ ^{(*)}$
$\mathrm{O_1 - 3\ O_3} = 2.3_7\ ^{(*)}, 3.1_2, 3.0_7$
$\mathrm{O_1 - 3\ O_4} = 2.3_8\ ^{(*)}, 2.5_8, 3.1_7$

$\mathrm{O_1 - O_5} = 2.8_8$
$\mathrm{O_1 - 2\ O_6} = 2.6_2, (3.3_4)$
$\mathrm{O_2 - O_3} = 2.3_6\ ^{(*)}$
$\mathrm{O_2 - 2\ O_4} = 2.4_5\ ^{(*)}\ 3.1_8$
$\mathrm{O_2 - 2\ O_5} = 2.7_8, 3.0_9$
$\mathrm{O_2 - 3\ O_6} = 2.7_0, 2.7_8, (3.4_2)$
$\mathrm{O_3 - 3\ O_4} = 2.4_2\ ^{(*)}, 2.7_8, 3.1_0$
$\mathrm{O_3 - 2\ O_5} = 2.6_3\ ^{(*)}, 3.2_1$
$\mathrm{O_3 - O_6} = 2.9_1$
$\mathrm{O_4 - 2\ O_5} = 3.0_1, (3.3_5)$
$\mathrm{O_4 - O_6} = 3.2_0$
$\mathrm{O_5 - 2\ O_5} = 2.7_3$
$\mathrm{O_5 - O_6} = 3.2_3$
$\mathrm{O_6 - 2\ O_6} = 3.0_4$

* These distances were taken from previously determined structures and used directly in the determination of the oxygen positions.

The accuracy of the distances Ce–O and S–O is assumed to be about ±0.1 Å, but the distances between oxygen atoms (not belonging to the same sulphate group) are somewhat more uncertain, probably ±0.2–0.3 Å.

The O–O distances are all within the normal range, showing that the lattice is also supported by O–O contacts.

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SUMMARY

The crystal structure of a new cerium oxy sulphate, $CeOSO_4H_2O$, has been determined. It has an orthorhombic unit cell with the dimensions:

$$a = 11.98_3 \pm 0.01\ \text{Å}$$
$$b = 8.26_7 \pm 0.01\ \text{Å}$$
$$c = 4.33_2 \pm 0.01\ \text{Å}$$
$$V = 429.1\ \text{Å}^3$$

The unit cell contains four formula units.

A structure is proposed with atomic positions in the space group $D_2^4 - P\,2_12_12_1$. The positions of the cerium and sulphur atoms were determined with Fourier methods. The parameters of the oxygen atoms were determined partly from electron density projections and partly from geometrical considerations, using interatomic distances from previously determined structures.

The cerium atoms are arranged in zigzag rows (*cf.* Fig. 8) parallel to the c axis and with rather short Ce – Ce distances (3.58 Å). In the rows they are joined by O^{2-} ions, each of which is in contact with three cerium atoms. The resulting $(CeO^{2+})_n$ strings are then connected by sulphate groups and water molecules, so that a coordination Ce – 8 O is obtained.

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The crystal structure of $NaSbF_4$

By ANDERS BYSTRÖM, SIW BÄCKLUND, and KARL-AXEL WILHELMI

With 5 figures in the text

A number of papers dealing with the crystal chemistry of complex antimony(III)-fluorides have been published previously (1, 2, 3, 4).

One characteristic feature in the structures of both K_2SbF_5 (3) and $KSbF_4$ (4) is the five-coordinate antimony atoms—present in K_2SbF_5 as isolated SbF_5^{2-}-complexes, and in $KSbF_4$ as tetranuclear $(Sb_4F_{16})^{4-}$-complexes. The opinion that the coordination number of antimony in these compounds is five is supported by the fact that the compounds of the isomorphous series R_2SbF_5 ($R = K$, Rb, Cs, NH_4, Tl) represent the phases with the largest number of fluorine atoms per antimony atom in the corresponding systems RF-SbF_3. However, FLÜCKIGER (5) described a compound possessing the formula Na_3SbF_6, and this may possibly contain six-coordinate antimony atoms. An investigation of the compounds in the system NaF-SbF_3 was therefore carried out.

FLÜCKIGER claimed to have prepared Na_3SbF_6 by dissolving Sb_2O_3 and NaF in the required proportions in hydrofluoric acid and crystallizing at room temperature. In this way he obtained prismatic crystals which he considered to be Na_3SbF_6. In attempts to produce the same compound, we dissolved Sb_2O_3 and Na_2CO_3 in the proportions 1:3 in excess dilute hydrofluoric acid. Two compounds crystallized from the solution, first $NaHF_2$ and then a complex antimony fluoride, which separated as prismatic crystals. The latter compound was $NaSbF_4$ as was apparent from the analysis:

observed	Na: 10.4%, Sb: 55.1 %, F: 34.2;%
calculated for $NaSbF_4$	Na: 10.4%, Sb: 55.15%, F: 34.4%

If the solution was evaporated to dryness at 100°, another complex compound was formed, and this could be recrystallized from aqueous solution at room temperature (thus without excess HF). However, the antimony content of this compound was 46.3%, which corresponds closely to the formula Na_2SbF_5 ($Sb = 46.33\%$) and this phase cannot possibly consist of Na_3SbF_6 ($Sb = 39.95\%$). Attempts to produce Na_3SbF_6 from melts have also failed. The compounds $NaSbF_4$ and Na_2SbF_5 both decompose at higher temperatures according to the equations:

$$2\ NaSbF_4 \rightarrow Na_2SbF_5(s) + SbF_3(l)\ (250°\ C)$$
$$Na_2SbF_5 \rightarrow 2\ NaF(s) + SbF_3(g)\ (300\text{–}350°\ C)$$

It seems that FLÜCKIGER'S Na_3SbF_6-phase thus actually consisted of a compound with lower NaF content. The crystals are reported by FLÜCKIGER to have a high reflecting power and this accords well with the appearance of the crystals of Na_2SbF_5 which we obtained. We can, however, conclude from our investigation that a compound with a higher NaF content than Na_2SbF_5 probably does not exist in the system.

Unit cell and symmetry of $NaSbF_4$

Rotation and Weissenberg photographs of $NaSbF_4$ showed that the symmetry is monoclinic with the *b*-axis along the prism-axis. The unit cell dimensions, derived from powder photographs (Cr-K $\lambda_a = 2{,}2909$ Å) in focusing cameras are:

$$a = 8.06 \pm 0.01 \text{ Å}, \; b = 5.52 \pm 0.01 \text{ Å}, \; c = 8.56 \pm 0.01 \text{ Å} \; \beta = 94.1° \pm 0.2°$$

The density was found to be 3.83, which agrees well with the calculated value of 3.85 assuming 4 $NaSbF_4$ in the unit cell.

$0k0$ reflexions are present only when $k = 2n$, and $h0l$ reflexions only when $l = 2n$, with the exception of a very weak reflexion $10\bar{3}$. The space group may thus be C_{2h}^5–$P2_1/c$, or (if $10\bar{3}$ is considered) C_{2h}^2–$P2_1/m$ or C_2^2–$P2_1$. As will be seen, an arrangement which fits very well with the intensities of the observed reflexions was found in C_{2h}^5–$P2_1/c$. The presence of $10\bar{3}$ may indicate that this arrangement is in some way an idealisation of the true structure. The true space group cannot be C_{2h}^2, because in that case the Sb atoms must be situated in the mirror planes, leading to intensities not in agreement with the observed ones. Thus the space group would be C_2^2–$P2_1$ and distortion would lead to a non-centro symmetric structure. However this distortion, if it exists, must be rather small, as the only apparent manifestation of it is the weak $10\bar{3}$ reflexion.

Physical properties of $NaSbF_4$

Some optical measurements on $NaSbF_4$ were carried out by Dr N. SUNDIUS at the Geological Survey. The following refractive indices were found:

$n_a = 1.475$
$n_\beta = 1.521$ (β parallel to b)
$n_\gamma = 1.540$

The crystals are thus optically negative. $2V_a$ is fairly large and amounts to 70°–80°. As will be seen, the birefringence is not large enough to allow any predictions concerning the atomic arrangements.

Good cleavages could be obtained along (100) and (001) but not along (010). The cleavage along (001) may be somewhat easier to obtain than that along (100).

Intensity formula

In previous papers, *F*-values have been deduced from the visually estimated intensities using the formula: $F_{\text{obs}} = \sqrt{\frac{I_{\text{obs}}}{PL}}$, where PL represents the combined Lorentz-polarization factor. This definition of F_{obs} has been retained also in this paper. For the $0kl$ and $hk0$ reflexions, corrections for absorption have been carried out. These new sets of F_{values} are designated F_{corr} in Table 2 and calculated by the formula:

Table 1

$NaSbF_4$. Comparison between observed and calculated F-values for $h0l$ reflexions. Cu-K-radiation.

$h\,k\,l$	F_{obs}	F_{calc}	$h\,k\,l$	F_{obs}	F_{calc}	$h\,k\,l$	F_{obs}	F_{calc}
1 0 0	30	− 58	0 0 4	160	−181	$2\,0\,\bar{6}$	50	68
2 0 0	110	−139	1 0 4	30	50	$3\,0\,\bar{6}$	60	61
3 0 0	110	126	2 0 4	40	59	$4\,0\,\bar{6}$	120	−117
4 0 0	140	−142	3 0 4	70	−109	$5\,0\,\bar{6}$	—	− 4
5 0 0	100	− 91	4 0 4	100	119	$6\,0\,\bar{6}$	60	44
6 0 0	140	149	5 0 4	60	71	$7\,0\,\bar{6}$	100	− 69
7 0 0	—	− 1	6 0 4	140	−134	$8\,0\,\bar{6}$	50	41
8 0 0	70	− 53	7 0 4	80	39	0 0 8	80	68
9 0 0	120	87	8 0 4	—	13	1 0 8	90	− 91
10 0 0	40	− 31	9 0 4	120	− 92	2 0 8	50	47
0 0 2	60	− 90	$1\,0\,\bar{4}$	—	31	3 0 8	60	80
1 0 2	70	−104	$2\,0\,\bar{4}$	120	131	4 0 8	90	− 87
2 0 2	80	120	$3\,0\,\bar{4}$	90	− 98	5 0 8	—	2
3 0 2	30	− 36	$4\,0\,\bar{4}$	—	9	6 0 8	60	47
4 0 2	50	− 55	$5\,0\,\bar{4}$	120	105	7 0 8	80	− 70
5 0 2	140	123	$6\,0\,\bar{4}$	130	−118	$1\,0\,\bar{8}$	—	− 5
6 0 2	—	− 19	$7\,0\,\bar{4}$	—	− 8	$2\,0\,\bar{8}$	130	−124
7 0 2	140	−103	$8\,0\,\bar{4}$	130	109	$3\,0\,\bar{8}$	80	91
8 0 2	100	70	$9\,0\,\bar{4}$	100	− 92	$4\,0\,\bar{8}$	60	39
9 0 2	—	− 6	0 0 6	90	92	$5\,0\,\bar{8}$	100	− 75
10 0 2	100	− 77	1 0 6	70	62	$6\,0\,\bar{8}$	80	78
$1\,0\,\bar{2}$	80	168	2 0 6	130	−155	0 0 10	90	− 91
$2\,0\,\bar{2}$	60	− 87	3 0 6	60	60	1 0 10	—	3
$3\,0\,\bar{2}$	30	− 48	4 0 6	30	39	2 0 10	90	99
$4\,0\,\bar{2}$	110	148	5 0 6	100	−134	3 0 10	70	− 63
$5\,0\,\bar{2}$	100	− 79	6 0 6	60	76	4 0 10	—	− 21
$6\,0\,\bar{2}$	—	− 14	7 0 6	80	69	$10\,1\,\bar{0}$	80	66
$7\,0\,\bar{2}$	140	99	8 0 6	90	− 87	$20\,1\,\bar{0}$	—	− 3
$8\,0\,\bar{2}$	140	− 92	9 0 6	40	49	$30\,1\,\bar{0}$	80	− 91
$9\,0\,\bar{2}$	50	− 33	$1\,0\,\bar{6}$	110	− 96			

$$F_{corr} = \sqrt{\frac{I_{obs}}{ATPL}} \quad \text{with} \quad A = \int\limits_V \frac{e^{-\mu x}\,dv}{V} \quad \text{and} \quad T = e^{-2B\left(\frac{\sin\theta}{\lambda}\right)^2}$$

The absorption coefficients have been calculated according to HOWELLS (6). The value of B in the temperature factor was empirically obtained from the decrease of the quotient $\frac{F_{corr}\cdot\sqrt{I}}{F_{obs}}$ with increasing $\sin^2\theta$ and chosen to be 0.75, thus assuming B to have the same value for all reflexions.

The improvement in agreement after the application of absorption and temperature factors is demonstrated by the decrease in the reliability index

$$R = \frac{\Sigma\,||F_{obs}|-|F_{calc}||}{\Sigma\,|F_{obs}|},$$

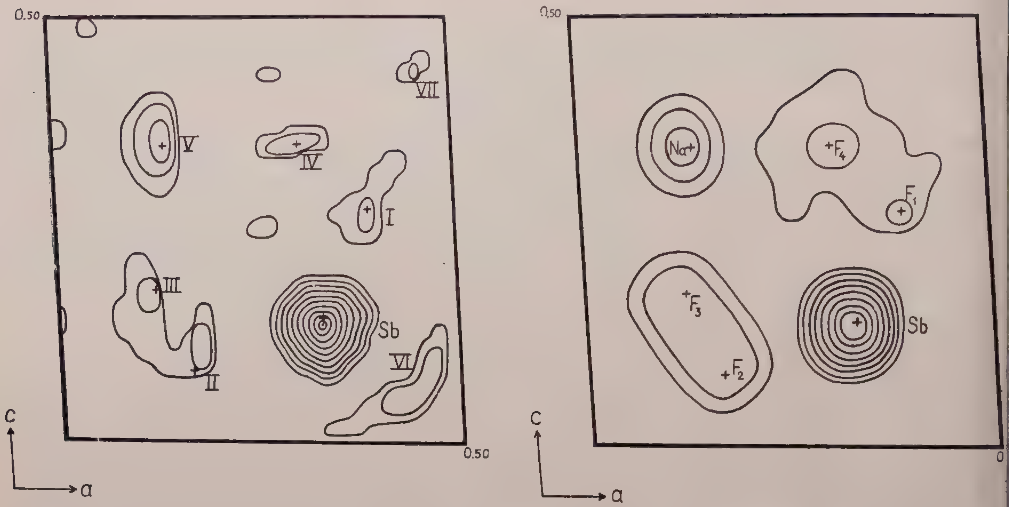

Fig. 1. $NaSbF_4$. Projection of the electron density on (010). In a) the summation is carried out with the F_{obs} terms and in b) with the terms F_{obs} multiplied with the factor $e^{-4\left(\frac{\sin\theta}{\lambda}\right)^2}$ (see the text page 4). In b) only every secoud level line of the Sb maxima is shown.

when the F_{corr} values are used instead of the F_{obs} values:

		F_{obs}	F_{corr}
$0\,k\,l$	$R=$	0.295	0.125
$h\,k\,0$	$R=$	0.269	0.170

(Suitable scale factors were used, so that in each case

$$\Sigma|F_{obs}|=\Sigma|F_{corr}|=\Sigma|F_{calc}|.)$$

For the $h0l$ reflexions which are not corrected for absorption, R is 0.202. The largest absorption occurs for reflexions at low glancing angles. This is demonstrated by the fact that R for $h0l$ reflexions with $h<5$ and $l<4$ is 0.376 for $h\geq 5$ and $l\geq 4$ 0.172.

Determination of the structure

The positions of the four antimony atoms were deduced from trial and error calculations. The signs of the amplitudes of almost all of the reflexions could then be deduced. The electron density projection in the xz plane was prepared (Fig. 1 a) using the F_{obs} $(h0l)$ values. Seven minor peaks were obtained (labelled I-VII in the figure). As the scattering factors of Na and F do not differ greatly, no distinction between these two kinds of atoms were made at first. Trial and error calculations showed that a satisfactory agreement between observed and calculated intensities was obtained only with fluorine and sodium atoms in the positions I-V. The maxima VI and VII might be due to spurious diffraction effects. A new electron density projection was prepared, using F-values multiplied by a factor $e^{-4\frac{\sin^2\theta}{\lambda^2}}$. In this projection the maxima VI and VII were considerably lower than the maxima I-V (Fig. 1 b), indicating that the former maxima were due largely to the finite limits of summation.

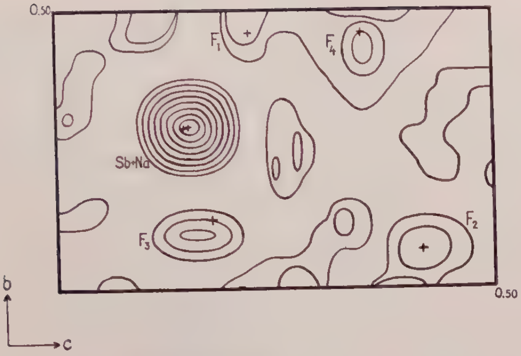

Fig. 2. $NaSbF_4$. Projection of the electron density on (100). In the (Sb + Na) maxima only every second level line is shown.

The Sb-F distances in K_2SbF_5 and $KSbF_4$ are 2.0–2.3 Å. It is therefore probable that the maximum V, which is somewhat higher than the maxima I-IV, corresponds to a sodium atom, as the distance from the nearest Sb-peak in the projection is 2.5 Å. With this reasonable assumption, it is possible to assign approximate x and z parameters to the sodium and the fluorine atoms, which will all be placed in the position 4(e) in C_{2h}^5–$P2_1/c$ like the antimony atoms. The x and z parameters were then refined by trial and error calculations on reflexions with high contributions from the Na and F atoms. In the table below, the influence of the light atoms is compared with that of the antimony atoms for some of these reflexions and as may be seen, a remarkable improvement in agreement results, when the influence of the light atoms is reckoned for. This justifies the chosen parameter values, which are shown in the summary of parameters on p. 7. As no correction for the absorption was carried out for the $h0l$ reflexions, the relative F-values are compared, the largest F-value in each group being given the arbitrary value 100. F_{Sb} denotes the structure amplitude of the antimony atoms and F_{calc} the amplitude when those of the sodium and fluorine atoms are added.

Approximate y parameters for the sodium and fluorine atoms could now be deduced on the assumptions that each fluorine atom should have at least one Sb contact with Sb–F 2.0–2.3 Å, that no F–F distances less than 2.4 Å should occur, and that the Na-F distances should be ~2.3 Å. Two different sets of y-parameters which fullfilled these conditions were found.

$h\,k\,l$	K $\vert F_{obs}\vert$	K $\vert F_{Sb}\vert$	K $\vert F_{calc}\vert$	$h\,k\,l$	K $\vert F_{obs}\vert$	K $\vert F_{Sb}\vert$	K $\vert F_{calc}\vert$
0 0 2	75	26	75	0 0 4	100	100	100
1 0 2	88	84	87	1 0 4	19	84	28
2 0 2	100	100	100	2 0 4	25	18	32
3 0 2	21	21	29	$2\,0\,\bar{4}$	100	100	100
4 0 2	36	94	45	$3\,0\,\bar{4}$	75	105	75
5 0 2	100	100	100	3 0 6	100	100	100
7 0 2	100	100	100	4 0 6	58	98	65
8 0 2	72	102	68	1 0 8	100	100	100
$7\,0\,\bar{2}$	100	100	100	2 0 8	56	22	52
$8\,0\,\bar{2}$	100	61	93				

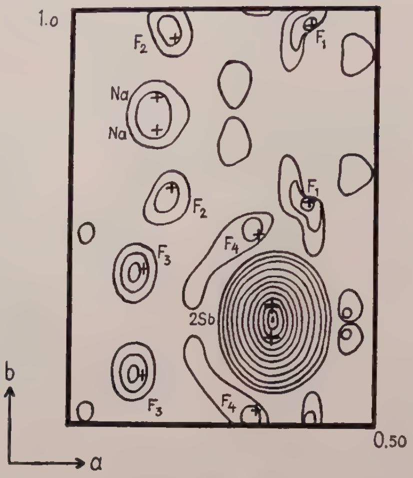

Fig. 3. $NaSbF_4$. Projection of the electron density on (001). In the Sb maxima only every second level line is shown.

	$2\pi y_{Na}$	$2\pi y_{F1}$	$2\pi y_{F2}$	$2\pi y_{F3}$	$2\pi y_{F4}$
I	−105°	−140°	0°	150°	140°
II	−105°	− 15°	−155°	45°	15°

Calculations showed that the Na and F atoms must have an influence of the same magnitude as those of the Sb atoms on the *h*11 reflexions. The *F*-values of these reflexions are compared below with those calculated for the two alternatives:

h k l	$\|F_{corr}\|$	$\|F_{calc}\|$ I	$\|F_{calc}\|$ II	*h k l*	$\|F_{corr}\|$	$\|F_{calc}\|$ I	$\|F_{calc}\|$ II
0 1 1	35	31	36	$\bar{1}$ 1 1	—	10	21
1 1 1	52	34	30	$\bar{2}$ 1 1	31	47	43
2 1 1	—	25	14	$\bar{3}$ 1 1	47	83	55
3 1 1	59	10	39	$\bar{4}$ 1 1	—	8	17
4 1 1	85	37	59	$\bar{5}$ 1 1	—	57	1
5 1 1	—	34	29	$\bar{6}$ 1 1	31	16	18
6 1 1	45	11	34	$\bar{7}$ 1 1	43	8	38
7 1 1	55	7	60	$\bar{8}$ 1 1	46	22	22
8 1 1	—	17	6	$\bar{9}$ 1 1	—	27	13
9 1 1	28	29	21				

As may be seen, alternative I leads to serious discrepancies (compare 211 and 311; 511, 611, and 711; 51$\bar{1}$, 61$\bar{1}$, and 71$\bar{1}$) whereas alternative II gives *F*-values which agree fairly well with those observed. This was further verified by comparison of the observed and calculated *F*-values for the 0*kl* and *hk*0 reflexions (Table II). It appeared that the index of reliability decreased considerably when the influence of the sodium and fluorine atoms were added to that of the metal atoms:

		Sb atoms only:	all atoms:
0 *k l*	*R* =	0.236	0.125
h k 0	*R* =	0.238	0.170
h 0 *l*	*R* =	0.275	0.202

(For *h*0*l* reflexions no correction for absorption is carried out, see p. 79).

The electron density projections on (100) and (001) are shown in Fig. 2 and 3. Maxima corresponding to the light atoms are found with parameter values corresponding rather well with those from the trial and error calculations. Some extra maxima are probably due to the low number of terms in these syntheses (see Table 2).

As may be seen from the table below, the structure amplitudes of the sodium and fluorine atoms are of the same magnitude as those of the antimony atoms for certain reflexions, thus providing strong evidence for the correctness of the suggested parameters:

$h\,k\,l$	$\lvert F_{\text{corr}}\rvert$	$\lvert F_{\text{Sb}}\rvert$	$\lvert F_{\text{calc}}\rvert$	$h\,k\,l$	$\lvert F_{\text{corr}}\rvert$	$\lvert F_{\text{Sb}}\rvert$	$\lvert F_{\text{calc}}\rvert$
3 0 0	120	165	126	1 3 0	83	97	84
4 0 0	115	65	142	2 3 0	128	92	113
5 0 0	87	77	91	1 5 0	31	41	27
1 2 0	56	72	41	2 5 0	64	39	54
2 2 0	66	74	60				
3 2 0	105	132	99				

$h\,k\,l$	$\lvert F_{\text{corr}}\rvert$	$\lvert F_{\text{Sb}}\rvert$	$\lvert F_{\text{calc}}\rvert$	$h\,k\,l$	$\lvert F_{\text{corr}}\rvert$	$\lvert F_{\text{Sb}}\rvert$	$\lvert F_{\text{calc}}\rvert$
0 0 2	91	35	90	0 0 8	64	77	68
0 1 2	145	168	200	0 1 8	110	77	93
0 4 2	29	14	24	0 2 8	66	68	70
0 5 2	28	45	28	0 2 9	57	42	64
0 1 4	91	54	91	0 3 9	27	5	17
0 2 4	124	115	128	0 4 9	52	70	65
0 0 6	109	68	92				
0 1 6	123	101	102				

The finally accepted parameter values are:

$C_{2h}^5 - P2_1/c$ All atoms in 4(e): $x\,y\,z$

	$2\pi x$	$2\pi y$	$2\pi z$	x	y	z
4 Sb	119°	103°	50.5°	0.331 ± 0.0015	0.286 ± 0.003	0.140 ± 0.0015
4 Na	50°	−105°	−55°	0.139 ± 0.010	-0.292 ± 0.015	-0.153 ± 0.010
4 F_1	140°	−15°	100°	0.389 ,,	−0.042 ,,	0.278 ,,
4 F_2	60°	−155°	30°	0.167 ,,	−0.431 ,,	0.083 ,,
4 F_3	45°	45°	65°	0.125 ,,	0.125 ,,	0.181 ,,
4 F_4	110°	15°	−55°	0.306 ,,	0.042 ,,	−0.153 ,,

The interatomic distances are:

$Sb - 2F_1 = 2.19$ and 2.51 Å
$Sb - F_2 = 2.08$ Å
$Sb - F_3 = 1.93$ Å
$Sb - F_4 = 2.03$ Å

$Na - F_1 = 2.33$ Å
$Na - 2F_2 = 2.19$ and 2.59 Å
$Na - 2F_3 = 2.31$ and 2.33 Å
$Na - F_4 = 2.28$ Å

The shortest F–F distances are: F_1–F_3 = 2.41 Å, F_2–F_4 = 2.52 Å, F_2–F_3 = 2.62 Å, and F_3–F_4 = 2.69 Å. The F–F distances between different chains (see below) are considerably longer, the shortest being F_1–F_2 = 3.28 Å. The shortest Sb–Sb distances are 4.23 Å (within the chain) and 4.30 Å (between different chains).

The structure is shown in Fig. 4. Each antimony atom is bonded to five fluorine atoms, which surround the antimony atom in the same peculiar manner as in K_2SbF_5 and $KSbF_4$: the fluorine atoms occupy five of the six corners in a distorted octahedron with the antimony atom displaced from the center in a direction away from the five fluorine atoms (see Fig. 4). The polyhedra do not form closed complexes as in $KSbF_4$, but are joined to infinite chains of the formula $[SbF_4]_\infty$, running in the *b*-direction. The mean Sb–F distance for the three unshared F atoms is 2.02 Å (±0.10 Å) which is very close to the corresponding distances in K_2SbF_5 (2.03 Å) and in $KSbF_4$ (2.01 Å). The Sb–F distances in the Sb-F_1–Sb bridges differ rather much (2.19 and 2.51 Å) but the mean distance 2.35 Å, is within the limits of the errors the same as the corresponding distance in $KSbF_4$ (2.24 Å). Unfortunately, the parameters of the F_1 atoms are difficult to fix and the corresponding Sb–F distances are probably not accurate to more than ±0.1–0.2 Å. However, if the x parameter of these atoms is increased to 0.40, the corresponding distances are 2.21 and 2.43 Å, and the distances can be made still more nearly equal by small changes in the y and z parameters. In fact these changes in the parameter would

Table 2

Comparison between observed and calculated F-values. $0kl$ and $hk0$ reflexions. F_{corr} calculated according to the formula on p. 79. Cu-K-radiation.

$h\,k\,l$	$\lvert F_{\text{corr}}\rvert$	F_{calc}	$h\,k\,l$	$\lvert F_{\text{corr}}\rvert$	F_{calc}
0 2 0	99	−118	0 1 5	18	20
0 4 0	89	86	0 2 5	77	− 65
0 6 0	25	− 29	0 3 5	—	− 32
0 1 1	33	− 36	0 4 5	94	87
0 2 1	75	58	0 5 5	—	14
0 3 1	68	61	0 6 5	97	−109
0 4 1	100	− 76	0 0 6	109	92
0 5 1	85	− 67	0 1 6	123	102
0 6 1	102	109	0 2 6	82	− 78
0 7 1	65	73	0 3 6	86	− 93
0 0 2	91	− 90	0 4 6	36	− 33
0 1 2	145	−200	0 5 6	29	28
0 2 2	22	17	0 1 7	—	− 2
0 3 2	145	136	0 2 7	—	4
0 4 2	29	− 29	0 3 7	91	92
0 5 2	28	− 25	0 4 7	—	9
0 6 2	—	0	0 5 7	94	− 95
0 1 3	20	14	0 0 8	64	68
0 2 3	19	17	0 1 8	110	− 93
0 3 3	99	− 84	0 2 8	66	− 70
0 4 3	65	− 69	0 3 8	58	68
0 5 3	114	104	0 4 8	36	54
0 6 3	41	36	0 1 9	22	− 12
0 0 4	167	−181	0 2 9	57	64
0 1 4	91	93	0 3 9	27	− 17
0 2 4	124	127	0 4 9	52	− 65
0 3 4	54	− 24	0 0 10	107	− 91
0 4 4	59	− 68	0 1 10	46	− 49
0 5 4	—	13	0 2 10	74	73
0 6 4	29	22			

$h\ k\ l$	$\lvert F_{corr} \rvert$	F_{calc}	$h\ k\ l$	$\lvert F_{corr} \rvert$	F_{calc}
1 0 0	70	− 58	8 2 0	60	41
2 0 0	132	−139	9 2 0	105	− 99
3 0 0	120	126	1 3 0	83	84
4 0 0	115	−142	2 3 0	128	−113
5 0 0	87	− 91	3 3 0	–	− 5
6 0 0	115	149	4 3 0	72	65
7 0 0	–	− 1	5 3 0	91	− 68
8 0 0	47	− 53	6 3 0	–	21
9 0 0	103	87	7 3 0	102	76
10 0 0	29	− 31	8 3 0	77	− 69
1 1 0	100	−137	9 3 0	–	− 29
2 1 0	114	166	0 4 0	60	86
3 1 0	–	20	1 4 0	58	− 49
4 1 0	134	−154	2 4 0	39	− 45
5 1 0	87	87	3 4 0	91	83
6 1 0	–	14	4 4 0	50	− 31
7 1 0	110	− 97	5 4 0	24	− 35
8 1 0	93	95	6 4 0	78	65
9 1 0	35	26	7 4 0	–	− 20
10 1 0	93	− 87	8 4 0	29	− 39
0 2 0	85	−118	1 5 0	31	− 27
1 2 0	56	41	2 5 0	64	54
2 2 0	66	60	3 5 0	–	23
3 2 0	105	− 99	4 5 0	53	− 59
4 2 0	62	58	5 5 0	–	17
5 2 0	97	78	6 5 0	–	8
6 2 0	110	−102	7 5 0	25	− 48
7 2 0	39	29			

also increase the somewhat short F_1–F_2 distances. It is possible that in the determination of the F_1 parameters the errors in the other parameters overlap, so that the somewhat less good agreement between observed and calculated *F*-values which is obtained if the suggested adjustments to the parameters are made is quite accidental and not a consequence of an increased error in the F_1 parameters.

The suggested structure obviously explains very well the cleavages of the crystals along (0 0 1) and (1 0 0). These planes only cut off two of the comparatively weak Na–F bonds per unit area. The good cleavage along (1 0 0) supports the opinion that the next shortest Sb–F contact, operating between the Sb atoms in one double chain and the F_4 atoms in another and 2.85 Å in length, corresponds to a bond of secondary order (compared with the five other Sb–F bonds).

The errors in the interatomic distances

As in the case of K_2SbF_5 (3) and $KSbF_4$ (4) the errors in the individual Sb–F distances are large, of the order of ±0.1–0.2 Å. An accuracy of 0.01–0.05 Å would be desirable in order to discuss the different Sb–F distances within the complex groups in the compounds. In the actual case of $NaSbF_4$, the light atoms, Na + F, contribute about 45% of the electrons in the unit cell. However, their influence on the *F*-values is considerably less: the mean value for the $h0l$, $hk0$, and $0kl$ reflexions is 25%. The mean *F*-value is 70. The errors in the Sb parameters are estimated to be ±0.0015 in the *a*- and *c*-direction, and ±0.003 in the *b*-direction, correspond-

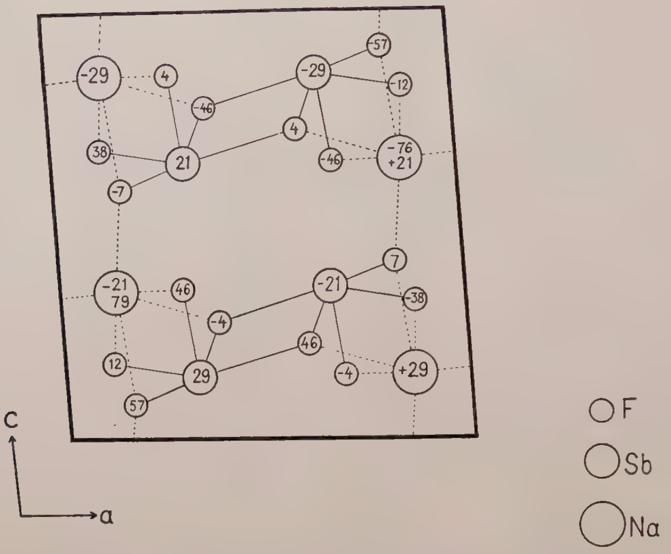

Fig. 4. $NaSbF_4$. Projection of the structure on (010). Compare Fig. 5.

ing to ±0.01 Å in all three directions. The error in the observed F-values after correction for absorption and application of temperature factor, is probably 10–15% (as indicated by the reliability index, p. 7), that is 7–10 units in the mean F-value. The mean value of F_{Na+F} is 18. It would be necessary to estimate the F-values to at least an accuracy of 10–15% of the F_{Na+F}-values (or probably less because of the larger number of parameters), i.e. to 2–3 units in the F-values, thus to an accuracy of 3–5% to decrease the errors in the fluorine parameters to ±0.01 Å. This is probably beyond the accuracy of visual estimation of X-ray reflexions. Another possible way to determine the Sb–F distances more exactly is to deduce the F parameters from a three-dimensional Fourier synthesis. However, this would also necessitate the calculation of absorption coefficients for all the reflexions involved in the synthesis and the time necessary for this is probably considerably longer than that for the three-dimensional synthesis, even if this is made without any device for summation.

It is possible to reduce the absorption corrections by using spherical crystals. We have tried to prepare such crystals, and have succeeded in the case of some dichromates which are under investigation. However, apparently the complex antimony fluorides have too low cohesion, and in this case the crystals broke up into fragments.

Other complex phases in the system NaF-SbF_3

At least three more phases exist in the system NaF–SbF_3, two with a NaF content lower than that of $NaSbF_4$ and one with a higher NaF content.

As mentioned above, the latter has the composition Na_2SbF_5. This compound does not belong to the previously investigated isomorphous series R_2SbF_5 (3). Approximative cell dimensions of Na_2SbF_5 are:

$$a = 8.1\ \text{Å},\ b = 5.4\ \text{Å},\ c = 11.2\ \text{Å}$$

and there are four formula units in the cell.

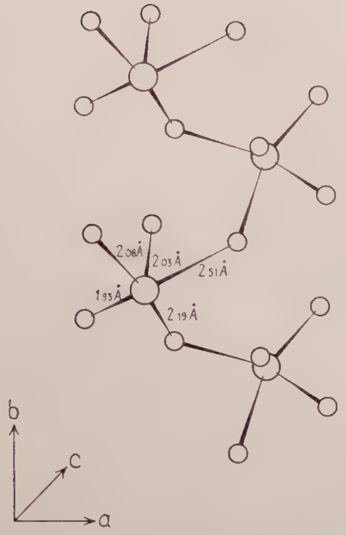

Fig. 5. $NaSbF_4$. Part of an infinite chain of the composition $(SbF_4^-)_\infty$.

Acknowledgement

We are indebted to Dr. N. Sundius for the optical measurements on $NaSbF_4$.

This investigation has been financially supported by the Swedish Natural Science Research Council. One of the authors (A. B.) is indebted to the Lars Hiertas Minne Foundation and to the Royal Swedish Academy of Science for grants.

SUMMARY

The crystal structure of $NaSbF_4$ has been determined. The structure is built up by double chains $[SbF_4^-]_\infty$ running in the b-direction of the monoclinic cell which has the cell dimensions: $a = 8.06 \pm 0.01$ Å, $b = 5.52 \pm 0.01$ Å, $c = 8.56 \pm 0.01$, $\beta = 94.1°$. The space group is C_{2h}^5—$P2_1/c$ and the atomic coordinates are given on p. 7.

Some results from an investigation of other sodium antimony(III)-fluorides are given.

University of Stockholm, Institute of Inorganic and Physical Chemistry, February 1952.

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On the structures of antimony (III) oxidehalides.[1] II

The crystal structure of SbOCl

By MAJA EDSTRAND

With 12 figures in the text

This paper forms part of an investigation on the antimony (III) oxidehalides undertaken in the hope of getting some idea of the bond character of antimony in these compounds. In $Sb_4O_5Cl_2$ and the isomorphous $Sb_4O_5Br_2$ EDSTRAND (1947) it seems impossible to say if the bonds are mainly covalent or mainly ionic.

Preparation

SbOCl has been prepared by several authors as indicated in Gmelins Handbuch (1949). In the present investigation SbOCl has been prepared as described by SABANEJEW (1871) by hydrolysis of $SbCl_3$ with water, and by SCHAEFFER's method (1869) which involves hydrolysis of $SbCl_3$ with absolute ethanol at higher temperatures.

$SbCl_3$ was hydrolysed with water in molecular proportions varying between 1 : 2.5 and 1 : 15, both at room temperature and at about 50° C. The time varied between ten days and three months. The precipitates were filtered off, dried in a desiccator over concentrated H_2SO_4 and then washed free of adhering $SbCl_3$ with ether. The substances were crystalline, the crystals having a rhombohedral, almost cubic appearance. These crystals were, however, too small to give good single crystal photographs. SbOCl was therefore also prepared by hydrolysing $SbCl_3$ with an equimolecular amount of absolute ethanol in sealed Pyrex tubes at about 150° C during 10 to 20 hours. In this case the precipitates were directly washed with ether, and although the crystals differed somewhat in shape from those obtained by hydrolysis with water, they were sufficiently large to give good single crystal photographs. SbOCl samples prepared in these two ways gave identical powder photographs.

Analyses

Antimony was determined by titration with 0.1 *N* $KBrO_3$[2]. The oxidechloride was dissolved in 25 % HCl and titrated at about 60° C using methyl red as indicator. At the equivalence point the solution became colourless. This method was used for one sample (1). For the others a method described by SMITH and MAY (1941) which is a modification of this bromate method, was used.

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[1] Part I. M. EDSTRAND, Acta Chem. Scand. *1*, 178–203 (1947).

[2] J. M. KOLTHOFF, Die Massanalyse, Berlin (1928) p. 443.

The titration was carried out at room temperature in solutions containing 5–35 % concentrated HCl and with naphthol blue-black as irreversible indicator. This gave a very sharp end-point.

In one case (1) the halogen was determined by boiling the chloride with Na_2CO_3 solution in a platinum dish, filtering, acidifying the filtrate with HNO_3 and precipitating the chloride with $AgNO_3$. The silver halide was then filtered off, dried, and weighed.

In the other cases, the oxidechloride was dissolved in a tartaric acid solution and then titrated by the Volhard[1] method in the presence of nitrobenzene with 0.1 *M* $AgNO_3$ and 0.1 *M* NH_4CNS.

	Antimony %	Chlorine %
calc. for SbOCl	70.29	20.47
found for substance after hydrolysis		
with water (1)	69.9	20.67
water (2)	70.64	19.93
ethanol (3)	70.84	20.10

Unit cell and space group

Single crystals were selected and set and rotation photographs round the three axes were taken. The following Weissenberg photographs were taken with Cu-radiation: $0kl$, $1kl$, $2kl$, $3kl$, $4kl$, $5kl$, $h0l$, $h1l$, $h2l$, $h3l$, $h4l$, $h5l$, $h6l$, and $hk0$. To be sure not to miss the innermost reflections two Weissenberg photographs namely $0kl$ and $h0l$ were taken with Cr-radiation. The crystals proved to be monoclinic. It was impossible to determine the cell dimensions accurately from ordinary powder photographs, as they were very rich in lines, but through the kindness of Dr. ARNE MAGNÉLI of the University of Uppsala, we obtained a Guinier photograph with Cr-radiation in which the lines were separated at low θ-values (see table 1). This gave the following cell dimensions:

$$\begin{aligned} a &= 9.52 \text{ kX} = 9.54 \text{ Å} \\ b &= 10.75 \text{ kX} = 10.77 \text{ Å} \\ c &= 7.92 \text{ kX} = 7.94 \text{ Å} \\ \beta &= 103.6^\circ \\ V &= 786.96 \text{ kX}^3 = 791.77 \text{ Å}^3 \end{aligned}$$

with an accuracy of about 0.2 %.

If twelve formula units are assumed per unit cell the density would be

$$d_{\text{calc}} = 4.36 \qquad d_{\text{obs}} = 4.31,\ 4.31$$

(The density of the crystals was determined from the loss of weight in benzene.).

The above values for the axes lead to the axial ratios 0.8856 : 1 : 0.7367 which are fairly near those given by COOKE (1877) viz. 0.8936 : 1 : 0.7587 with $\beta = 76^\circ 31'$ ($103^\circ 29'$).

In the Weissenberg photographs all reflections $h0l$ with h odd and all reflections $0k0$ with k odd are absent. This is characteristic of the space group $C_{2h}^5 - P\,2_1/a$.

Patterson syntheses to find the antimony positions

As antimony is much heavier than the other atoms in the cell it seemed appropriate to apply Patterson syntheses to find the positions of the twelve antimony atoms.

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[1] I. M. KOLTHOFF and E. B. SANDELL, Textbook of Quantitative Inorganic Analysis, New York (1938) p. 544.

Table 1

Guinier photograph of SbOCl CrKα-radiation. $\lambda = 2.2863$ kX = 2.2910 Å.

Notations: st = strong, m = medium, w = weak, vw = very weak.

h k l	$10^4 \sin^2\theta$ calc	$10^4 \sin^2\theta$ obs	*I* obs	*h k l*	$10^4 \sin^2\theta$ calc	$10^4 \sin^2\theta$ obs	*I* obs
0 0 1	0221			3 2 $\bar{1}$	1789		
1 1 0	0266	0265	m	0 4 0	1811		
0 1 1	0334	0333	w +	3 2 0	1828	1826	w
1 1 $\bar{1}$	0400	0401	w	2 0 2	1840	1841	w
0 2 0	0453	0455	vw	3 1 $\bar{2}$	1852		
1 1 1	0573			1 3 $\bar{2}$	1881		
1 2 0	0605	0603	m	0 3 2	1901		
2 0 0	0611			2 1 2	1954		
2 0 $\bar{1}$	0659	0660	w +	1 4 0	1963		
0 2 1	0673			3 1 1	1969		
2 1 0	0725			0 0 3	1986		
1 2 $\bar{1}$	0740			1 1 $\bar{3}$	1992	1994	w
2 1 $\bar{1}$	0772			2 3 1	2024		
0 0 2	0883			0 4 1	2031	2029	vw
1 2 1	0913	0912	w	2 0 $\bar{3}$	2078	2078	m
1 1 $\bar{2}$	0976	0975	vw	1 4 $\bar{1}$	2097		
0 1 2	0996 }	1002	w + +	0 1 3	2099		
2 0 1	1005 }			2 3 $\bar{2}$	2166		
2 2 0	1064	1063	vw	2 1 $\bar{3}$	2191		
2 2 $\bar{1}$	1111	1108	st	3 2 $\bar{2}$	2191		
2 1 1	1118	1119	w −	1 3 2	2227		
2 0 $\bar{2}$	1148			1 4 1	2271		
1 3 0	1171	1170	w +	2 2 2	2293		
0 3 1	1239			3 2 1	2309 }	2315	w
2 1 $\bar{2}$	1261			4 0 $\bar{1}$	2320 }		
1 3 $\bar{1}$	1305	1303	w	1 2 $\bar{3}$	2332	2331	w
1 2 $\bar{2}$	1315			3 3 $\bar{1}$	2355		
1 1 2	1322	1323	w	3 3 0	2394		
0 2 2	1335	1336	m	2 4 0	2422 }	2428	w
3 1 $\bar{1}$	1450	1450	w	4 1 $\bar{1}$	2433 }		
2 2 1	1458			0 2 3	2439		
1 3 1	1479			4 0 0	2445		
3 1 0	1489			2 4 $\bar{1}$	2469		
2 2 $\bar{2}$	1600			1 1 3	2512		
2 3 0	1630	1635	m +	2 2 $\bar{3}$	2531		
1 2 2	1661	1659	w + +	4 1 0	2559		
2 3 $\bar{1}$	1677	1676	w −	4 0 $\bar{2}$	2635		

The values of the intensities used were estimated visually from the Weissenberg photographs and then corrected for the Lorentz and polarization factors. For the non-equatorial zones the curves given by KAAN and COLE (1949) were used. These corrected intensities were used instead of the F^2 values.

In space group $C_{2h}^5 - P\,2_1/a$ we have the following point positions $2(a)\ 000;\ \tfrac{1}{2}\tfrac{1}{2}0$
$(b)\ 00\tfrac{1}{2};\ \tfrac{1}{2}\tfrac{1}{2}\tfrac{1}{2}$ $(c)\ \tfrac{1}{2}00;\ 0\tfrac{1}{2}0$ $(d)\ \tfrac{1}{2}0\tfrac{1}{2};\ 0\tfrac{1}{2}\tfrac{1}{2}$
$4(e)\ xyz;\ \bar{x}\bar{y}\bar{z};\ \tfrac{1}{2}-x,\ \tfrac{1}{2}+y,\ \bar{z};\ \tfrac{1}{2}+x,\ \tfrac{1}{2}-y,\ z$

Because of the symmetry of the space group the parameters of $4(e)$ need only be considered within the limits $0 \leq x \leq \tfrac{1}{2}$, $0 \leq y \leq \tfrac{1}{2}$, and $0 \leq z \leq 1$. In the Patterson space $P(uvw)$ these point positions give the following vectors:

I vectors within a twofold position of weight 2 $(\tfrac{1}{2}\tfrac{1}{2}0)$

II vectors between two twofold positions of weight 4

$$2(a)-2(b) \text{ and } 2(c)-2(d)\ (0\,0\,\tfrac{1}{2});\ (\tfrac{1}{2}\,\tfrac{1}{2}\,\tfrac{1}{2})$$
$$2(a)-2(c) \text{ and } 2(b)-2(d)\ (\tfrac{1}{2}\,0\,0);\ (0\,\tfrac{1}{2}\,0)$$
$$2(a)-2(d) \text{ and } 2(b)-2(c)\ (\tfrac{1}{2}\,0\,\tfrac{1}{2});\ (0\,\tfrac{1}{2}\,\tfrac{1}{2})$$

Vectors within a fourfold position

$$\text{III of weight } 1 \pm (2x, \pm 2y,\ 2z)$$
$$\text{IV of weight } 2 \pm (\tfrac{1}{2}+2x,\ \tfrac{1}{2},\ 2z)$$
$$\pm (\tfrac{1}{2},\ \tfrac{1}{2}+2y,\ 0)$$

V vectors between two fourfold positions with indices i and j of weight 2

$$\pm[x_i - x_j \quad \pm(y_i - y_j),\quad z_i - z_j]$$
$$\pm[x_i + x_j \quad \pm(y_i + y_j),\quad z_i + z_j]$$
$$\pm[\tfrac{1}{2} + x_i + x_j, \tfrac{1}{2} \pm (y_i - y_j),\quad z_i + z_j]$$
$$\pm[\tfrac{1}{2} + x_i - x_j, \tfrac{1}{2} \pm (y_i + y_j),\quad z_i - z_j]$$

It seems most likely that the twelve antimony atoms occupy three fourfold positions. This would give Patterson projections very crowded in maxima. The vectors of group IV, however, would in the projection $P(uvp)$ give maxima at $u = \frac{1}{2}$, $v = \frac{1}{2} + 2y$ and at $u = \frac{1}{2} + 2x$, $v = \frac{1}{2}$ and would in the projection $P(pvw)$ give maxima at $v = \frac{1}{2}$, $w = 2z$, and $v = \frac{1}{2} + 2y$, $w = 0$. Therefore the two projections $P(uvp)$ and $P(pvw)$ were calculated. It was found, however, that only the projection $P(uvp)$ could be interpreted at this point of the investigation. In order to obtain the z-parameters the cut $P(u\frac{1}{2}w)$ was calculated, as only the vectors of IV $\pm(\frac{1}{2} + 2x,\ \frac{1}{2},\ 2z)$ are bound to fall here, and the other vectors only incidentally can occur here depending on the y-parameter.

The $P(uvp)$-projection

On account of the symmetry it is only necessary to calculate the area $0 \leq u \leq \frac{1}{2}$, $0 \leq v \leq \frac{1}{2}$.

The $P(uvp)$-projection really found is given in Fig. 1, which is referred to in the following discussion.

Antimony in twofold positions would require a maximum at $u = \frac{1}{2}$, $v = \frac{1}{2}$. As $P(uvp)$ actually has a minimum at this point it seems likely that the twofold positions can be excluded.

We thus need only consider the vectors from the fourfold positions, and start with the vectors of group IV. At $v = \frac{1}{2}$ we have the following maxima $u = \frac{1}{2} + 2x \approx 0.05$ (low), 0.35 (high), 0.65 (high), 0.95 (low), and so on. With $0 \leq x \leq \frac{1}{2}$ we get the following possible parameters $x \approx 0.075$ (A), 0.225 (B), 0.275 (B), 0.425 (A). The x-values derived from the high maxima are indicated by A and those from the low maxima by B.

At $u = \frac{1}{2}$ we have the following maxima: $v = \frac{1}{2} + 2y \approx 0$, 0.17 (low), 0.27 (high), 0.35 (low), 0.45, 0.55, 0.65 (low), 0.73 (high), 0.83 (low), 1.0 a.s.o. With $0 \leq y \leq \frac{1}{2}$ we get the following possible parameters $y \approx 0.025$, 0.075, 0.12, 0.17, 0.25, 0.33, 0.38, 0.425, 0.475.

In order to choose a set of three x-parameters and three y-parameters among the given x and y-values we must also consider the vectors of the groups III and V which in this projection are:

$$\text{III of weight } 1 \pm (2x_i,\ \pm 2y_i) \qquad i = 1, 2, 3$$
$$\text{V of weight } 2 \pm [x_i - x_j,\ \pm(y_i - y_j)] \quad i, j = 1, 2, 3 \qquad i \neq j$$

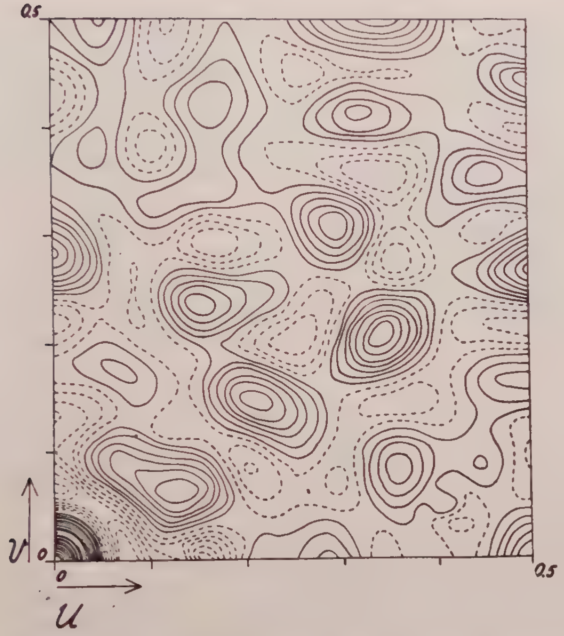

Fig. 1. $P(uvp)$. Contours at an interval of 50 arbitrary units. Negative values dotted.

$$\begin{array}{ll} \pm [x_i + x_j, & \pm (y_i + y_j)] \\ \pm [\tfrac{1}{2} + x_i + x_j, & \tfrac{1}{2} \pm (y_i - y_j)] \\ \pm [\tfrac{1}{2} + x_i - x_j, & \tfrac{1}{2} \pm (y_i + y_j)] \end{array}$$

When we choose three x-values out of the given four this implies that two x must belong either to group A or B. As the maximum from which A is derived has a height that is almost double the height of the maximum from which B is derived, it is likely that two belong to A. Therefore we call those from A $x_1 \approx 0.425$ and $x_2 \approx 0.075$, which means that $x_1 + x_2 = \frac{1}{2}$. Because of this the possibility $x_1 = x_2$ does not give new combinations, as we have both x and $\frac{1}{2} - x$ in the fourfold position 4(e). These two x-values require maxima at $\pm u = x_1 - x_2 \approx 0.35$, $\pm u = x_1 + x_2 \approx 0.50$ and $\pm u = \frac{1}{2} + x_1 + x_2 \approx 1.0$ where also maxima can be found. x_3 must now be either 0.225 or 0.275. However, it is not possible to decide between these two values of x_3 as for both of them maxima are found at $u = x_1 - x_3, x_2 - x_3, x_1 + x_3, x_2 + x_3$, which, however, never can be 0, 0.5 or 1.0.

The expected maximum for $\pm u = \frac{1}{2} + x_1 + x_2 \approx 1.0$ means that $\pm v = \frac{1}{2} \pm (y_1 - y_2) \approx 0, 0.28, 0.50$ (very low), 0.72, 1.0. This gives $|y_1 - y_2| \approx 0, 0.22, 0.50$. We can expect maxima for $[u = x_1 - x_2,\ v = \pm (y_1 - y_2)] \approx [0.35, 0]$ (does not exist); [0.35, 0.22] (exists) and [0.35, 0.50] (exists, but can be explained in another way). $|y_1 - y_2| \approx 0.5$ would require one $y = 0$ and one $y = 0.5$ with $0 \leq y \leq \frac{1}{2}$ but these are not found in the given series of y-values. It is thus likely that $|y_1 - y_2| \approx 0.22$.

The expected maximum for $\pm u = x_1 + x_2 \approx 0.50$ means that $\pm v = y_1 + y_2 \approx 0$, 0.17 (low), 0.27 (high), 0.35 (low), 0.45, 0.55, 0.65 (low), 0.73 (high), 0.83 (low), 1.0. With $0 \leq y \leq \frac{1}{2}$ we can exlude $y_1 + y_2 = 0$ and 1.0. When $x_1 + x_2 \approx 0.50$ the four vectors $\pm [x_1 + x_2,\ \pm (y_1 + y_2)]$ of weight two coincide to give two vectors [0.5,

$\pm(y_1+y_2)]$ of weight four, which make it probable that the highest peaks are explained by these vectors so that $y_1+y_2\approx 0.27$ or 0.73. The combination $|y_1-y_2|\approx 0.22$, $y_1+y_2\approx 0.27$ gives $y_1\approx 0.245$ $y_2\approx 0.025$, and the combination $|y_1-y_2|\approx 0.22$, $y_1+y_2\approx 0.73$ gives $y_1\approx 0.475$, $y_2\approx 0.255$. All these y-values fall within the series given on p. 4. It should be observed that as $|y_1-y_2|$ has been used the indices of x and y are not necessarily the same. One y-value is obviously about 0.25 and the other one is either 0.025 or 0.475.

With these parameters it is possible to explain the maxima at $u=\frac{1}{2}$ with $v\approx 0\,(1.0)$, $0.27\,(0.73)$, $0.45\,(0.55)$. We now desire to find y_3. As the maximum at $v\approx 0.35$ does not seem to have its centre on $u=\frac{1}{2}$ we need not consider it here. The remaining maxima then give $v=\frac{1}{2}+2y_3\approx 0.83$, or 1.17. This gives $y_3\approx 0.165$, or 0.335.

We can now combine the parameters in the following way

$$\text{a}\qquad \begin{cases} x_1\approx 0.425\\ y_1\approx 0.25\end{cases}\qquad \begin{cases} x_2\approx 0.075\\ y_2\approx 0.025,\ 0.475\end{cases}\qquad \begin{cases} x_3\approx 0.225,\ 0.275\\ y_3\approx 0.165,\ 0.335\end{cases}$$

$$\text{b}\qquad \begin{cases} x_1\approx 0.425\\ y_1\approx 0.025,\ 0.475\end{cases}\qquad \begin{cases} x_2\approx 0.075\\ y_2\approx 0.25\end{cases}\qquad \begin{cases} x_3\approx 0.225,\ 0.275\\ y_3\approx 0.165,\ 0.335\end{cases}$$

The vectors $\pm[x_1-x_3,\ \pm(y_1-y_3)]$ and $\pm[x_2-x_3,\ \pm(y_2-y_3)]$ can only be satisfactorily explained with the following combinations

$$\text{a}_1\qquad \begin{cases} x_1\approx 0.425\\ y_1\approx 0.25\end{cases}\qquad \begin{cases} x_2\approx 0.075\\ y_2\approx 0.025\end{cases}\qquad \begin{cases} x_3\approx 0.275\\ y_3\approx 0.165\end{cases}$$

$$\text{a}_2\qquad \begin{cases} x_1\approx 0.425\\ y_1\approx 0.25\end{cases}\qquad \begin{cases} x_2\approx 0.075\\ y_2\approx 0.475\end{cases}\qquad \begin{cases} x_3\approx 0.275\\ y_3\approx 0.335\end{cases}$$

$$\text{b}_1\qquad \begin{cases} x_1\approx 0.425\\ y_1\approx 0.025\end{cases}\qquad \begin{cases} x_2\approx 0.075\\ y_2\approx 0.25\end{cases}\qquad \begin{cases} x_3\approx 0.225\\ y_3\approx 0.165\end{cases}$$

$$\text{b}_2\qquad \begin{cases} x_1\approx 0.425\\ y_1\approx 0.475\end{cases}\qquad \begin{cases} x_2\approx 0.075\\ y_2\approx 0.25\end{cases}\qquad \begin{cases} x_3\approx 0.225\\ y_3\approx 0.335\end{cases}$$

All these four possibilities explain the maxima in the uv-projection quite satisfactorily. The xy-projections of them become identical if a coordinate transformation involving a translation of the coordinates $\frac{1}{2}$ along one or two of the axes is performed. We have thus in reality only *one* projection.

The cut $P(u\frac{1}{2}w)$

The intensities of the different Weissenberg photographs along the b-axis including the zones 0 to 6 were correlated, after correction for the Lorentz and polarization factors, by the aid of the Weissenberg photographs $0kl$ and $2kl$.

The $P(u\frac{1}{2}w)$ cut was calculated for $0\leq u\leq 1$ and $0\leq w\leq\frac{1}{2}$ (Fig. 2). From the symmetry, the whole area is easily obtainable.

As has been found from $P(uvp)$, the twelve antimony atoms are situated in three fourfold positions. This means that the vectors of group IV $\pm(\frac{1}{2}+2x,\ \frac{1}{2},\ 2z)$ will give us three maxima in the calculated area, but there are actually five high maxima. We have, however, found that one y-parameter is about 0.25. This means that for this fourfold position the four vectors $\pm(2x,\ \pm 2y,\ 2z)$ of weight 1 will give two vectors of weight 2 in $P(u\frac{1}{2}w)$, one of which falls within the calculated area, so that this position will give us maxima for both $\pm(\frac{1}{2}+2x,\ 2z)$ and $\pm(2x,\ 2z)$, that is

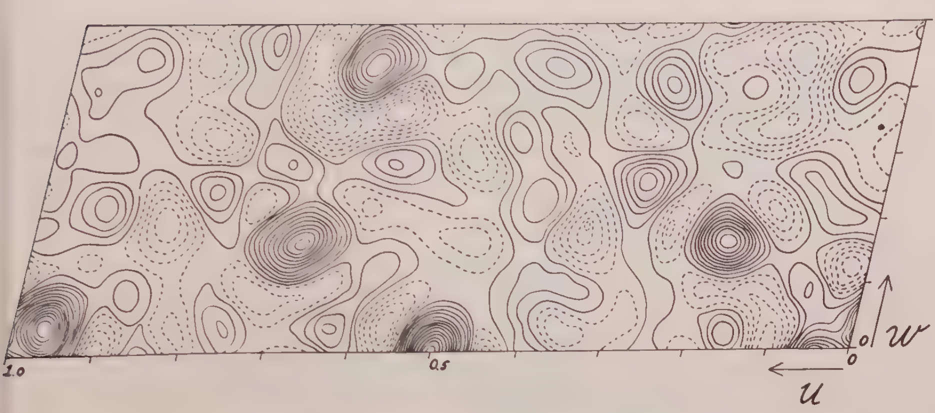

Fig. 2. $P(u\frac{1}{2}v)$. Contours at an interval of 500 arbitrary units. Negative values dotted.

two maxima with the same w and with u differing by $\frac{1}{2}$. This also can be seen in Fig. 2.

We have also found that one y-parameter is either about 0.025 or 0.475. This gives to the vector of group IV $\pm(\frac{1}{2}, \frac{1}{2}+2y, 0)$ a value of either $\pm(\frac{1}{2}, 0.55, 0)$ or $\pm(\frac{1}{2}, 1.45, 0)$. These v-values are so near $\frac{1}{2}$ or $\frac{3}{2}$ that we can expect a maximum in $P(u\frac{1}{2}w)$ at $(\frac{1}{2}\frac{1}{2}0)$ which is also found. There are also some lower maxima of a fairly considerable height. We can, however, certainly expect diffraction effects depending on the limits of summation and the uncertainty in the estimated intensities. These lower maxima were not taken into account, in the search of the antimony positions, and it also proved to be the case that all the high maxima in $P(u\frac{1}{2}v)$, $P(uvp)$, and $P(pvw)$ could be satisfactorily explained without considering them.

As the peak $(\frac{1}{2}\frac{1}{2}0)$ seems to be well accounted for we have only to consider the following four maxima: C: $u=0.965$, $w=0.043$, D_1: $u=0.687$, $w=0.168$, D_2: $u=0.177$, $w=0.167$, E: $u=0.644$, $w=0.438$. From what has been stated above (p. 6) it is clear that D_1 and D_2 come from the fourfold position with $y\approx 0.25$ and that only one of them need be considered when we choose the parameters.

From considerations of symmetry we can derive other maxima from C, D_1, D_2 and E. We need only consider the maxima within $0.5\leq u\leq 1.5$ and $0\leq w\leq 2$, when we are searching for the parameters from the vectors $u=\frac{1}{2}+2x_i$, $w=2z_i$.

We thus have the following series of maxima:

C : $(u, w) = (0.965, 0.043), (0.965, 1.043), (1.035, 0.957), (1.035, 1.957)$
D_1: $(u, w) = (0.687, 0.168), (0.687, 1.168), (1.313, 0.832), (1.313, 1.832)$
D_2: $(u, w) = (0.823, 0.833), (0.823, 1.833), (1.177, 0.167), (1.177, 1.167)$
E : $(u, w) = (0.644, 0.438), (0.644, 1.438), (1.356, 0.562), (1.356, 1.562)$

This gives the following series of x- and z-parameters

C : $(x, z)\approx(0.232, 0.022), (0.232, 0.522), (0.268, 0.478), (0.268, 0.978)$
D_1: $(x, z)\approx(0.093, 0.084), (0.093, 0.584), (0.407, 0.416), (0.407, 0.916)$
D_2: $(x, z)\approx(0.161, 0.416), (0.161, 0.916), (0.339, 0.084), (0.339, 0.584)$
E : $(x, z)\approx(0.072, 0.219), (0.072, 0.719), (0.428, 0.281), (0.428, 0.781)$

We now choose the combinations that have x-values which explain $P(uvp)$. These are x_1, $x_2\approx 0.425$, 0.075, $x_3\approx 0.225$, 0.275. Nearest to $x\approx 0.425$ come 0.428 from E and 0.407 from D_1. Nearest to $x \approx 0.075$ come 0.072 from E and 0.093 from D_1 Nearest to $x \approx 0.225$ comes 0.232 from C. Nearest to $x \approx 0.275$ comes 0.268 from C We now get the following possible combinations in space, with the more exact x-parameters from $P(u\tfrac{1}{2}w)$. From what has been stated above it is clear that only x-parameters from group D can be combined with $y \approx 0.25$. The group notations are the same as for $P(uvp)$.

$$a_1 \quad \begin{cases} x_1 \approx 0.407 \\ y_1 \approx 0.25 \\ z_1 \approx 0.416,\ 0.916 \end{cases} \quad \begin{cases} x_2 \approx 0.072 \\ y_2 \approx 0.025 \\ z_2 \approx 0.219,\ 0.719 \end{cases} \quad \begin{cases} x_3 \approx 0.268 \\ y_3 \approx 0.165 \\ z_3 \approx 0.478,\ 0.978 \end{cases}$$

$$a_2 \quad \begin{cases} x_1 \approx 0.407 \\ y_1 \approx 0.25 \\ z_1 \approx 0.416,\ 0.916 \end{cases} \quad \begin{cases} x_2 \approx 0.072 \\ y_2 \approx 0.475 \\ z_2 \approx 0.219,\ 0.719 \end{cases} \quad \begin{cases} x_3 \approx 0.268 \\ y_3 \approx 0.335 \\ z_3 \approx 0.478,\ 0.978 \end{cases}$$

$$b_1 \quad \begin{cases} x_1 \approx 0.428 \\ y_1 \approx 0.025 \\ z_1 \approx 0.281,\ 0.781 \end{cases} \quad \begin{cases} x_2 \approx 0.093 \\ y_2 \approx 0.25 \\ z_2 \approx 0.084,\ 0.584 \end{cases} \quad \begin{cases} x_3 \approx 0.232 \\ y_3 \approx 0.165 \\ z_3 \approx 0.022,\ 0.522 \end{cases}$$

$$b_2 \quad \begin{cases} x_1 \approx 0.428 \\ y_1 \approx 0.475 \\ z_1 \approx 0.281,\ 0.781 \end{cases} \quad \begin{cases} x_2 \approx 0.093 \\ y_2 \approx 0.25 \\ z_2 \approx 0.084,\ 0.584 \end{cases} \quad \begin{cases} x_3 \approx 0.232 \\ y_3 \approx 0.335 \\ z_3 \approx 0.022,\ 0.522 \end{cases}$$

Each group has now eight combinations. However, half of them can be transformed into the other half by a translation of $\tfrac{1}{2}$ along the z-axis. Each group thus really involves only four combinations.

In order to decide between these sixteen combinations the vectors of group V were calculated for the vw-projection and compared with the $P(pvw)$, actually found (Fig. 3). It then appeared that only one combination from each one of the headgroups a_1, a_2, b_1, and b_2 was possible. These combinations are

$$a_1 \quad \begin{cases} x_1 \approx 0.407 \\ y_1 \approx 0.25 \\ z_1 \approx 0.416 \end{cases} \quad \begin{cases} x_2 \approx 0.072 \\ y_2 \approx 0.025 \\ z_2 \approx 0.219 \end{cases} \quad \begin{cases} x_3 \approx 0.268 \\ y_3 \approx 0.165 \\ z_3 \approx 0.978 \end{cases}$$

$$a_2 \quad \begin{cases} x_1 \approx 0.407 \\ y_1 \approx 0.25 \\ z_1 \approx 0.416 \end{cases} \quad \begin{cases} x_2 \approx 0.072 \\ y_2 \approx 0.475 \\ z_2 \approx 0.219 \end{cases} \quad \begin{cases} x_3 \approx 0.268 \\ y_3 \approx 0.335 \\ z_3 \approx 0.978 \end{cases}$$

$$b_1 \quad \begin{cases} x_1 \approx 0.428 \\ y_1 \approx 0.025 \\ z_1 \approx 0.281 \end{cases} \quad \begin{cases} x_2 \approx 0.093 \\ y_2 \approx 0.25 \\ z_2 \approx 0.084 \end{cases} \quad \begin{cases} x_3 \approx 0.232 \\ y_3 \approx 0.165 \\ z_3 \approx 0.522 \end{cases}$$

$$b_2 \quad \begin{cases} x_1 \approx 0.428 \\ y_1 \approx 0.475 \\ z_1 \approx 0.281 \end{cases} \quad \begin{cases} x_2 \approx 0.093 \\ y_2 \approx 0.25 \\ z_2 \approx 0.084 \end{cases} \quad \begin{cases} x_3 \approx 0.232 \\ y_3 \approx 0.335 \\ z_3 \approx 0.522 \end{cases}$$

A closer inspection of these groups shows that the structures they give all can be obtained from each other by simple transformations involving translations of the origin $\tfrac{1}{2}$ along the axes. The group b_1 was arbitrarily chosen.

The cut P(½v0)

In the hope of obtaining more exact y-parameters the cut $P(\tfrac{1}{2}v0)$, where the vectors $\pm(\tfrac{1}{2},\ \tfrac{1}{2}+2y,\ 0)$ fall, was calculated. The Weissenberg photographs $h0l$,

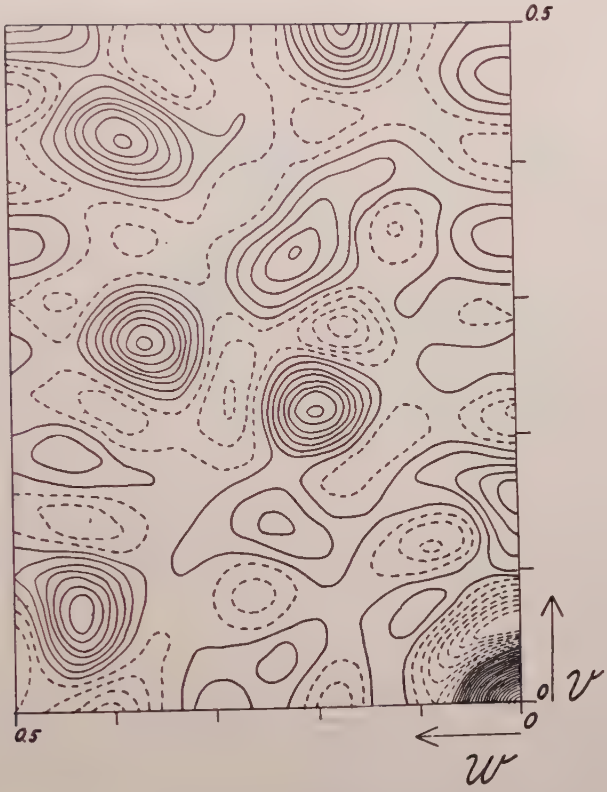

Fig. 3. $P(pvw)$. Contours at an interval of 50 arbitrary units. Negative values dotted.

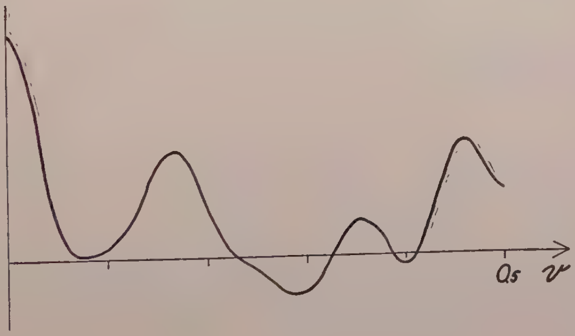

Fig. 4. $P(\frac{1}{2}v0)$.

$h1l$, $h2l$, $h3l$, $h4l$, $h5l$, $h6l$ were used for this purpose. In order to get factors for $7 \leq k \leq 13$ values were picked out from the photographs $0kl$, $1kl$, and $2kl$. The curve for $P(\frac{1}{2}v0)$ is given in Fig. 4. Here we find, as expected, three high maxima at 0, 0.165, and 0.460. From 0.460 we obtain $y_1 \approx 0.020$, from 0 $y_2 \approx 0.250$ and from 0.165 $y_3 \approx 0.167$. We thus have the following set of approximate parameters:

	x	y	z
Sb_1	0.428	0.020	0.281
Sb_2	0.093	0.250	0.084
Sb_3	0.232	0.167	0.522

Fourier syntheses

The best way to refine the antimony parameters obtained from the Patterson syntheses seemed to be by Fourier syntheses. It was also hoped that the Fourier syntheses would give an indication of the chlorine positions. An inspection of the different plane projections given by the preliminary antimony parameters showed that only the $\varrho(xyp)$-projection could be expected to be quite free from overlapping in the antimony positions.

The Fourier syntheses have been calculated with $|F_{\text{obs}}| = \sqrt{I \frac{1}{Lp}}$ where F is the structure factor, I is the estimated intensity, Lp is the combined Lorentz and polarization factors. In all syntheses the value of $F(000)$ has been omitted.

The ϱ (*xyp*)-projection

The first $\varrho(xyp)$ Fig. 5 was calculated with those values of $|F_{\text{obs}}|$ in which the signs seemed to be entirely determined by antimony. The antimony peaks were clearly resolved and with very small variations in their heights, so the proposed positions in the xy-projection seemed to be right except for small variations in the parameters. The maxima next in height were assumed to depend on the chlorine atoms. The attempts to refine both antimony and chlorine parameters from repeated computations of $\varrho(xyp)$ including more and more $|F_{\text{obs}}|$ values did not allow a certain determination of chlorine positions. Similarly a preliminary $\varrho(xpz)$ gave high maxima at the predicted antimony positions. There were, however, quite a lot of maxima next in height, that were not easily interpreted as chlorine positions. It seemed more appropriate to apply other Fourier syntheses and the bounded projection method was chosen.

The bounded projections $B_{x=0}^{x=\frac{1}{2}}$ and $B_{y=0}^{y=\frac{1}{2}}$ to refine the antimony parameters and find probable chlorine positions.

According to BOOTH (1948) p. 43 the bounded projection method gives, in suitably chosen cases, atomic coordinates with an accuracy not much inferior to that obtained by full three-dimensional Fourier methods. The computational work is, however, less.

If we project the electron density of the cell between the planes y_1' and y_2' on the $x'z'$-plane we obtain the bounded projection

$$B_{y_1'}^{y_2'} = \int_{y_1'}^{y_2'} \varrho(x' y' z')\, dy'$$

where the electron density for a cell with a symmetry centre has the form

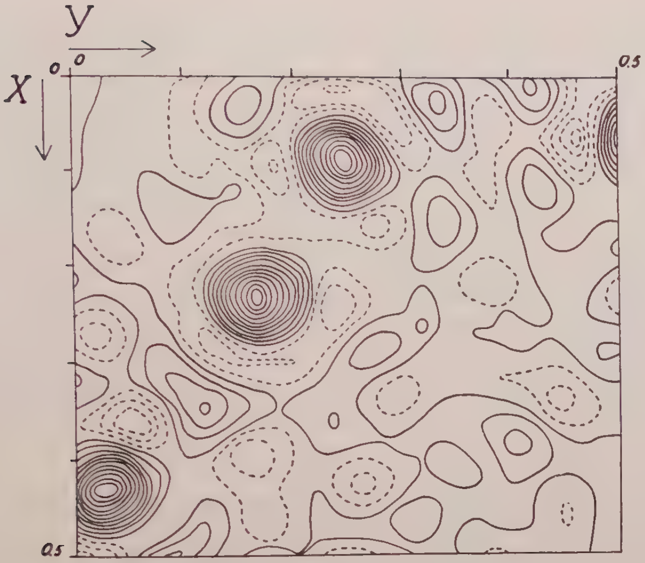

Fig. 5. Preliminary $\varrho(xyp)$. Contours at an interval of 100 arbitrary units. Negative values dotted.

$$\varrho(x'y'z') = \frac{1}{V}\sum_{h=-\infty}^{+\infty}\sum_{k=-\infty}^{+\infty}\sum_{l=-\infty}^{+\infty} F(hkl)\cos 2\pi\left(h\frac{x'}{a}+k\frac{y'}{b}+l\frac{z'}{c}\right).$$

This gives

$$B_{y_1'}^{y_2'} = \frac{1}{A}\sum\sum_{-\infty}^{+\infty} F(h0l)\cos 2\pi\left(h\frac{x'}{a}+l\frac{z'}{c}\right)\cdot\frac{1}{b}(y_2'-y_1') +$$

$$+\frac{1}{A}\sum\sum_{-\infty}^{+\infty}\sum\frac{1}{2\pi k}F(hkl)\cos 2\pi\left(h\frac{x'}{a}+l\frac{z'}{c}\right)\left(\sin 2\pi k\frac{y_2'}{b}-\sin 2\pi k\frac{y_1'}{b}\right)+$$

$$+\frac{1}{A}\sum\sum_{-\infty}^{+\infty}\sum\frac{1}{2\pi k}F(hkl)\sin 2\pi\left(h\frac{x'}{a}+l\frac{z'}{c}\right)\left(\cos 2\pi k\frac{y_2'}{b}-\cos 2\pi k\frac{y_1'}{b}\right).$$

From this it is clear that for a bounded projection all $F(hkl)$ terms are included. Now it is found, that if the limits are $y_1' = 0$ and $y_2' = \frac{b}{2}$ the expression is somewhat simplified and that only the zones $h0l$ and those with $k = 2n+1$ are involved. For the space group $C_{2h}^5 - P\ 2_1/a$, we also find that in the zones with $k = 2n+1$ we need only consider F-values with $h = 2n$. If we substitute $x = \frac{x'}{a}$, $y = \frac{y'}{a}$ and $z = \frac{z'}{a}$ we obtain the following expression:

$$B_{y=0}^{y=\frac{1}{2}} = \frac{1}{A}\sum_{h=0}^{\infty}\sum_{l=0}^{\infty}\{[F(h0l)+F(\bar{h}0l)]\cos 2\pi hx\cos 2\pi lz -$$

$$-[F(h0l)-F(\bar{h}0l)]\sin 2\pi hx\sin 2\pi lz\} -$$

$$-\frac{1}{A}\sum_{h=0}^{\infty}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\overset{\substack{h=2n\\k=2n+1}}{}\frac{4}{\pi k}\{[F(hkl)-F(\bar{h}kl)]\sin 2\pi hx\cos 2\pi lz+$$
$$+[F(hkl)+F(\bar{h}kl)]\cos 2\pi hx\sin 2\pi lz\}.$$

In a similar way we obtain the following expression for $B_{x=0}^{x=\frac{1}{2}}$ in space grou $C_{2h}^{5}-P\,2_1/a$.

$$B_{x=0}^{x=\frac{1}{2}}=\frac{2}{A}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\overset{k=2n}{}F(0kl)\cos 2\pi ky\cos 2\pi lz-$$

$$-\frac{2}{A}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\overset{k=2n+1}{}F(0kl)\sin 2\pi ky\sin 2\pi lz-$$

$$-\frac{2}{A}\sum_{h=0}^{\infty}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\overset{\substack{h=2n+1\\k=2n}}{}\frac{2}{\pi h}[F(hkl)+F(hk\bar{l})]\sin 2\pi ky\cos 2\pi lz-$$

$$-\frac{2}{A}\sum_{h=0}^{\infty}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\overset{\substack{h=2n+1\\k=2n+1}}{}\frac{2}{\pi h}[F(hkl)-F(hk\bar{l})]\cos 2\pi ky\sin 2\pi lz.$$

According to BOOTH (1948 p. 42) it is found that if either of the boundary plane passes through an area of positive electron density in a molecule, distortion of th resultant projection is liable to occur in this region. As for Sb_1 $y\approx 0.020$ it is likel that there will be distortions for $B_{y=0}^{y=\frac{1}{2}}$, and this must be borne in mind. Nevertheless the simplicity of the computations for $B_{y=0}^{y=\frac{1}{2}}$ makes it preferable to others involvin more work. $B_{y=0}^{y=\frac{1}{2}}$ is calculated for $0\leq x\leq\frac{1}{2}$, $0\leq z\leq 1$ and $B_{x=0}^{x=\frac{1}{2}}$ for $0\leq y\leq\frac{1}{2}$, $0\leq z\leq 1$ This gives in reality projections of the whole "asymmetric unit". In these bounde projections we can be sure that there will be no overlapping in the antimony position and hope that the chlorine positions also will be free from overlapping.

For the $B_{y=0}^{y=\frac{1}{2}}$ projection the Weissenberg photographs $h0l$, $h1l$, $h3l$ and $h5$ were used and correlated in the same way as for $P(u\frac{1}{2}w)$. For the $B_{x=0}^{x=\frac{1}{2}}$ projectio the Weissenberg photographs $0kl$, $1kl$, $3kl$ and $5kl$ were used and correlated wit the aid of $hk0$, $h1l$ and $h3l$.

For the first $B_{y=0}^{y=\frac{1}{2}}$ only those $|F_{\text{obs}}|$ whose signs seemed to depend entirely o antimony were used. This gave very clear antimony peaks, but the chlorine position seemed very uncertain. In the second $B_{y=0}^{y=\frac{1}{2}}$ the more exact antimony x- and z-para meters were used to determine the signs, but no assumption was made concerning th chlorine positions. In the second $B_{y=0}^{y=\frac{1}{2}}$ it appeared that the two maxima next i height to antimony were of a fairly regular shape and had almost the same height and it seemed quite probable that these indicated chlorine positions. There was third maximum, with a fairly regular shape, considerably lower than the first two but higher than the rest of the low maxima, which could indicate the third chlorin position.

At this stage the first $B^{x=\frac{1}{2}}_{x=0}$ was computed with the signs determined only by antimony. Here the antimony peaks were very clear and there appeared low maxima at approximately, the same z-values as for the probable chlorine positions in the second $B^{y=\frac{1}{2}}_{y=0}$, although only one of them was higher than a few other maxima, which might be only due to diffraction effects. On account of this only antimony was considered in the determination of the signs for the second $B^{x=\frac{1}{2}}_{x=0}$. In the second $B^{x=\frac{1}{2}}_{x=0}$ there now appeared three low clear maxima at these probable chlorine z-values. Here also the third peak was the lowest. Actually there was a fourth maximum at z about 0.5 which was a little higher than the third.

Now it seemed of interest to see what Sb-Cl and Cl-Cl distances these lower maxima would give. The exact coordinates of the maxima were determined by the interpolation table given by BOOTH (1948 p. 64). This gave the following parameters with x from the second $B^{y=\frac{1}{2}}_{y=0}$ and y, z from the second $B^{x=\frac{1}{2}}_{x=0}$ and all atoms in $4(e)$.

	x	y	z
Sb_1	0.427_7	0.024_2	0.281_2
Sb_2	0.089_3	0.242_3	0.081_2
Sb_3	0.231_8	0.172_8	0.521_5
Cl_1	0.402	0.405	0.119
Cl_2	0.364	0.353	0.586
Cl_3	0.344	0.115	0.900
?	?	0.069	0.517

It was found that the shortest distance between Sb_2 atoms and Cl_1 atoms was 2.46 Å and between Sb_3 atoms and Cl_2 atoms was 2.31 Å. As the distance Sb–Cl in $SbCl_3$ is 2.37 ± 0.02 kX in the gaseous state according to GREGG, HAMPSON, JENKINS, JONES, and SUTTON (1937), it seemed very likely that Cl_1 and Cl_2 were at approximately the correct positions. The shortest distances between Cl_3 atoms and Sb_1, Sb_2, and Sb_3, atoms were: Cl_3–Sb_1 3.11 and 3.25 Å, Cl_3–Sb_2 2.89 and 3.39 Å and Cl_3–Sb_3 3.02 Å. These distances are thus much longer than those for Cl_1–Sb_2 and Cl_2–Sb_3. They are however of approximately the same magnitude as in $Sb_4O_5Cl_2$ where the shortest Sb–Cl distance is 2.91 kX (EDSTRAND (1947)). At this point of the investigation it seemed likely that all the antimony atoms would have approximately the same coordination, as there are the same number of antimony, chlorine and oxygen atoms in the cell. This meant that it seemed more probable that the Cl_3–Sb_1 distance would be about 2.4 Å. As the peak for Cl_3 was the lowest chlorine maximum in both the second $B^{y=\frac{1}{2}}_{y=0}$ and the second $B^{x=\frac{1}{2}}_{x=0}$ it seemed likely that it would move more than the other maxima in the further refinement. It was also noticed that the shortest distance between Cl_3 and Cl_1 was 3.24 Å, which seemed very short compared with the sum of the ionic radii of about 3.6 Å.

It seemed of interest to find what value the x-coordinate of the maximum indicated by ? in the second $B^{x=\frac{1}{2}}_{x=0}$ would have, if it led to a chlorine position at reasonable distances from other atoms. The necessary conditions seemed to be: 1) its shortest distance from Sb_1 should not be longer than 2.4 Å, 2) it should not come nearer to Sb_3 than 2.3 Å, 3) the distance to a Cl of the same group should not be shorter than 3.6 Å. These conditions made it probable that $0.33 \leq x \leq 0.39$. However, there is no maximum in the second $B^{y=\frac{1}{2}}_{y=0}$ at $0.3 \leq x \leq 0.4$ and $z \approx 0.5$ of a height in any way comparable to that of Cl_3, so the proposed approximate position of Cl_3 really seems more likely.

Now the three maxima in the second $B_{x=0}^{x=\frac{1}{2}}$ next in height to the chlorine maxima seemed to be situated at quite probable oxygen positions, and cuts in the structure were considered at the corresponding y-values (0.07, 0.13 and 0.33) and it was found that there was indeed room for oxygen atoms.

At this stage it seemed most appropriate to calculate the third $B_{y=0}^{y=\frac{1}{4}}$ and also include the three chlorine positions in the determination of the signs. To speed up the computation the F_{calc} was approximated to

$$\tfrac{1}{4}\left[\sum_{1}^{3} A_{\text{Sb}_i} + \frac{f_{\text{Cl}}}{f_{\text{Sb}}}\sum_{1}^{3} A_{\text{Cl}_i}\right] \text{ where } A = 4\cos 2\pi\left(hx + lz + \frac{h+k}{4}\right)\cos 2\pi\left(ky - \frac{h+k}{4}\right)$$

and $\frac{f_{\text{Cl}}}{f_{\text{Sb}}} = 0.28$ for all reflections. The third $B_{y=0}^{y=\frac{1}{4}}$ is given in Fig. 6. We can see that the three chlorine peaks are now of nearly the same height. It may also be observed that Sb_1 with $y = 0.024$ gives a small maximum at $x \approx 0.07$, $z \approx 0.72$ as would be expected from the discussion on p. 12.

The x- and z-parameters from the third $B_{y=0}^{y=\frac{1}{4}}$ were used in the determination of the signs for the third $B_{x=0}^{x=\frac{1}{2}}$. The third $B_{x=0}^{x=\frac{1}{2}}$ found is given in Fig. 7, and the chlorine peaks seem now to be quite clear.

From the third $B_{y=0}^{y=\frac{1}{4}}$ and the third $B_{x=0}^{x=\frac{1}{2}}$ we get the following set of parameters where $z(y)$ indicates a z-value from $B_{y=0}^{y=\frac{1}{4}}$ and $z(x)$ a z-value from $B_{x=0}^{x=\frac{1}{2}}$.

	x	y	$z(y)$	$z(x)$	z(m. v.)
Sb_1	0.427_8	0.023_8	0.281_5	0.280_0	0.280_8
Sb_2	0.090_2	0.241_8	0.082_3	0.081_2	0.081_8
Sb_3	0.231_0	0.173_0	0.520_3	0.521_3	0.520_8
Cl_1	0.401_2	0.404_0	0.133_0	0.117_3	0.125_2
Cl_2	0.360_3	0.353_7	0.579_3	0.588_2	0.583_8
Cl_3	0.343_2	0.117_5	0.877_3	0.896_2	0.886_8

There still seems to be some diffraction effects in both $B_{y=0}^{y=\frac{1}{4}}$ and $B_{x=0}^{x=\frac{1}{2}}$. This might partly depend on absorption effects, which are certainly not negligible as will be discussed later on. However, the work involved in correcting for these effects in the whole series of $F(hkl)$ does not seem to justify such a refinement.

Discussion of the accuracy of the parameters and some of the distances from $B_{x=0}^{x=\frac{1}{2}}$ and $B_{y=0}^{y=\frac{1}{4}}$.

The maximum variation in the antimony z-parameters from $B_{x=0}^{x=\frac{1}{2}}$ and $B_{y=0}^{y=\frac{1}{4}}$ is 0.0015 which is equivalent to 0.012 Å. The maximum variation in the chlorine z-parameters from $B_{x=0}^{x=\frac{1}{2}}$ and $B_{y=0}^{y=\frac{1}{4}}$ is 0.019 which is equivalent to 0.15 Å. It then seems likely that the accuracy of the other antimony parameters is also about ± 0.0015 and that of the other chlorine parameters about ± 0.02.

From this it seems that the Sb–Cl distances might be accurate to within ± 0.3 Å and the Cl–Cl distance to within ± 0.5 Å. Now the shortest distance between Sb_2–Cl_1 is 2.47 Å and between Sb_3–Cl_2 2.29 Å. Within the limits of accuracy this indicates bonds of equal length. The shortest distances from Cl_3 to atoms from Sb_1, Sb_2, and

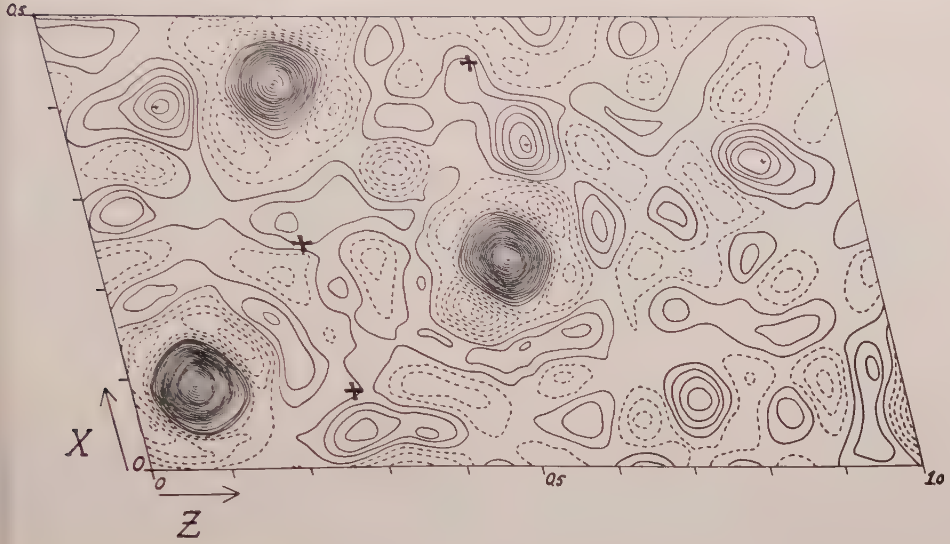

Fig. 6. The bounded projection $B_{y=0}^{y=\frac{1}{2}}$. Contours at an interval of 200 arbitrary units. Negative values dotted. The Sb and Cl positions are marked with small crosses, the O positions with large crosses.

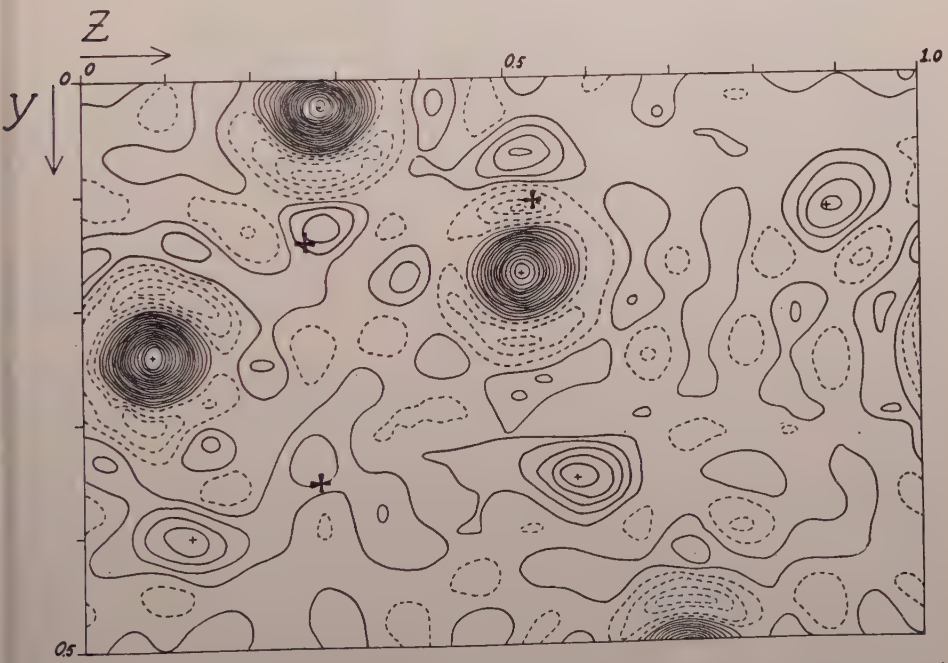

Fig. 7. The bounded projection $B_{x=0}^{x=\frac{1}{2}}$. Contours at an interval of 100 arbitrary units. Negative values dotted. The Sb and Cl positions are marked with small crosses, the O positions with large crosses.

Sb_3 are Cl_3–Sb_1 3.19, 3.20 Å, Cl_3–Sb_2 2.91 Å, and Cl_3–Sb_3 2.90 Å. This is a variation between 2.90 and 3.20 Å which, however, is not larger than the estimated accuracy. The comparatively large difference between the group 2.29, 2.47 and the group 2.90, 2.91, 3.19, 3.20 seems to be too great to indicate that all the Sb–Cl distances are indeed equal. The shortest Cl–Cl distances (except one) vary between 3.5 and 4.0 Å in good agreement with the sum of the ionic radii, 3.6 Å. One distance is 3.25 Å (Cl_1–Cl_3), but this is perhaps not so objectionable considering the low accuracy of these distances, and that Cl–Cl distances of 3.18 and 3.34 Å are present in the structures of $PdCl_2$ and $CuCl_2$ (Wells 1938, 1947). However admittedly the bonds in these compounds might be of a different type.

The oxygen positions

As mentioned on p. 102 there are minor maxima in the bounded projections that might indicate oxygen positions. Now the accuracy of the chlorine positions is not very great and oxygen positions taken from these projections would certainly be much less accurate. It therefore seemed better to try to locate the oxygen atoms from space considerations, and see if these positions corresponded to the low maxima in the projections.

It was assumed that the Sb–O distance would be 2.0 kX as in the two forms of Sb_2O_3 (Almin and Westgren (1942), Buerger and Hendricks (1938)), and that the sum of the ionic radii of oxygen and chlorine are

O–Cl	3.13 kX	(Goldschmidt),	3.21 kX	(Pauling)
O–O	2.64 »	»	2.80 »	»

(Values of ionic radii are taken from Internationale Tabellen (1935)).

Spheres of appropriate radii, inside of which no oxygen centres could occur, were drawn around the Sb and Cl centres. Cuts were made parallel with the xz-plane for $0 \leq y \leq \frac{1}{2}$ with intervals of 0.5 Å, which is equivalent to cuts with intervals of 0.25 Å for $0 \leq y \leq \frac{1}{4}$.

It seemed probable that every oxygen would be in contact with two antimony atoms as in the oxides, and for this reason positions which gave contact with only one antimony atom were rejected. Obviously the twelve oxygen atoms must belong to three fourfold positions.

It was found that there were only three independent domains in which the oxygens could be placed. However, these domains were somewhat extended in space and in two cases they were surrounded by three antimony atoms in a way that made it very difficult to choose only two of them as nearest neighbours to the oxygen atom. Fig. 8 shows cuts made at a) $y = 0.05$ and b) $y = 0.10$ to find a place for O_1. The possible position is bounded by the three Sb–O circles and the circles giving the forbidden area around the symmetry centre. We can see that O_1 always will come a little too close to one of the chlorine atoms. This is, however, assumed not to be serious because of slight inaccuracy in the chlorine parameters (p. 14). In Fig. 9 a and b, cuts are made at the finally accepted y-parameters for a) O_2 and b) O_3. As there seemed to be no special reason to prefer one set of two antimony atoms out of the three around O_1 and O_2, it was arbitrarily assumed that these oxygens were surrounded by three antimony at equal distances although this gave Sb–O distances greater than 2.0 Å.

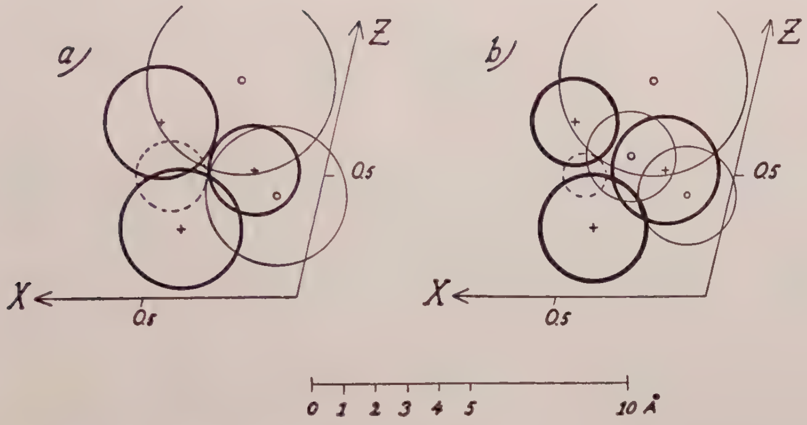

Fig. 8. Cuts giving the possible place for the oxygen O_1. a) at $y = 0.05$ b) at $y = 0.10$. + projection of centre of Sb, ∘ projection of centre of Cl. Thick circle = forbidden area around Sb, thin circle = forbidden area around Cl and dotted circle = forbidden area around symmetry centre.

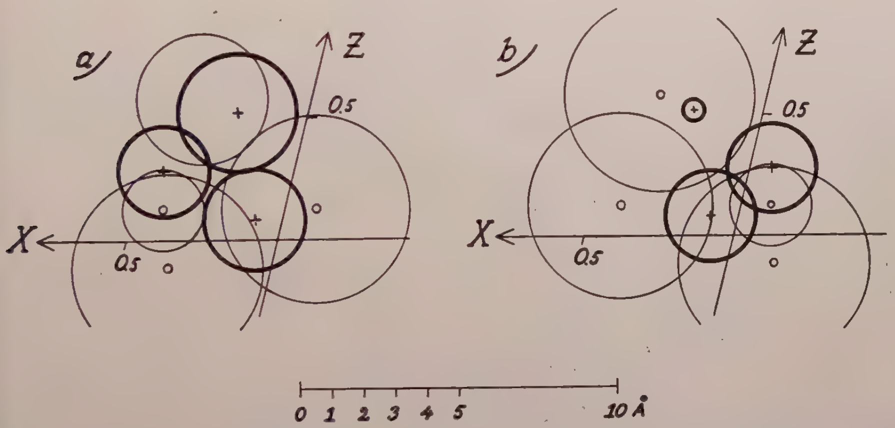

Fig. 9. a) Cut at $y = 0.144$ giving the possible place for the oxygen O_2. b) Cut at $y = 0.355$ giving the possible place for the oxygen O_3. + projection of centre of Sb. ∘ projection of centre of Cl. Thick circle = forbidden area around Sb, thin circle = forbidden area around Cl.

For O_1 it was assumed that it should be at the same distance from two atoms of Sb_1 and one atom of Sb_2, and that it should have a distance not less than 2.6 Å from its centrosymmetrically situated oxygen neighbour. This gave formally two solutions, one of which could be discarded later as it left no possible room for O_3.

For O_2 it was assumed that it should be at the same distance from one atom of each of Sb_1, Sb_2, and Sb_3 and that this distance should be the minimum distance. This gave formally only one solution.

O_3 seemed to be in contact with only two antimony, Sb_1 and Sb_2.

We then arrived at the following oxygen positions in 4(e).

	x	y	z
O_1	0.45_0	0.10_9	0.53_5
O_2	0.25_0	0.14_4	0.26_4
O_3	0.08_6	0.35_5	0.27_9

These oxygen positions are marked with large crosses in $B_{y=0}^{y=\frac{1}{2}}$ Fig. 6 and $B_{x=0}^{x=\frac{1}{2}}$ Fig. 7. We see that there are low maxima not far from these positions. The accuracy of these proposed oxygen parameters is very difficult to state. It seems likely that the oxygens are in the vicinity of these positions, although the number of antimony atoms really in contact with oxygen might be two instead of three.

The atomic coordinates of SbOCl with all atoms in 4(e) are then:

	x	y	z
Sb_1	0.427_8	0.023_8	0.280_8
Sb_2	0.090_2	0.241_8	0.081_8
Sb_3	0.231_0	0.173_0	0.520_8
Cl_1	0.40_1	0.40_4	0.12_5
Cl_2	0.36_0	0.35_4	0.58_4
Cl_3	0.34_3	0.11_8	0.88_7
O_1	0.45_0	0.10_9	0.53_5
O_2	0.25_0	0.14_4	0.26_4
O_3	0.08_6	0.35_5	0.27_9

with an accuracy of the parameters of about ± 0.0015 for Sb and ± 0.02 for chlorine.

F values were then calculated for $h0l$ and $0kl$ with the parameters of all atoms included.

$$\text{a)}\quad F_{\text{calc}} = \text{const} \cdot f_{\text{Sb}} \left[\sum_1^3 A_{\text{Sb}_i} + \frac{f_{\text{Cl}}}{f_{\text{Sb}}} \sum_1^3 A_{\text{Cl}_i} + \frac{f_{\text{O}}}{f_{\text{Sb}}} \sum_1^3 A_{\text{O}_i} \right]$$

but as the F_{calc} seemed to fall off too rapidly with increasing θ, they were also calculated as:

$$\text{b)}\quad F_{\text{calc}} = \text{const} \cdot \left[\sum_1^3 A_{\text{Sb}_i} + \frac{f_{\text{Cl}}}{f_{\text{Sb}}} \sum_1^3 A_{\text{Cl}_i} + \frac{f_{\text{O}}}{f_{\text{Sb}}} \sum_1^3 A_{\text{O}_i} \right]$$

which is a function in which F_{calc} falls off less rapidly although there is no special theoretical reason. f_{Sb}, f_{Cl} and f_{O} are the atomic scattering factors for Sb, Cl and O with due consideration taken to their variation with θ. It was found that the agreement between F_{obs} and F_{calc} was best for (b) which also was used in calculating tables 2 (for $h0l$) and 3 (for $0kl$). This indicates that there are appreciable absorption effects. Corrections for absorption would certainly improve the agreement and would perhaps also diminish the diffraction effects in $B_{x=0}^{x=\frac{1}{2}}$ and $B_{y=0}^{y=\frac{1}{2}}$. However, this has not been done as it seems that the bounded projections still give quite good information on the structure.

Discussion of the structure

Projections of the structure of SbOCl are given in Figs 10 and 11. They are projected along the x-axis on the yz-plane and along the z-axis on the xy-plane. In all projections

Table 2

Calculated and observed F-values of $h0l$ for a Weissenberg photograph of SbOCl. Cu$K\alpha$-radiation.

h	$h00$		$h01$		$h02$		$h03$		$h04$		$h05$		$h06$		$h07$		$h08$		$h09$		$h0\,10$	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
10	–2		37	17	39	55	82	89														
8	–10		–47	77	92	109	–28		39	35	25	32										
6	–118	128	31	47	–19	23	20	43	–45	58	140	136	–3		21	28						
4	–23	39	–36	40	1		–92	58	73	55	–13		36	44	–56	48	112	104				
2	2		53	42	–85	71	–24	16	–34	39	–16		–89	116	59	77	19	32	48	47		
0					26	29	–30	32	33	45	–122	128	–52	59	4		16		–49	69		
–2			45	43	–16	14	125	84	–14		3		–13	23	10		–85	107	–72	68	10	
4			–95	62	48	32	26	17	55	38	24		107	80	2		–59	61	–23		–52	39
6			–27	23	9		–8	12	–55	29	63	47	57	37	9		7	22	71	63		
8			–7	14	17	24	–59	88	–58	37	25	28	–40	14	–14	13	38	52	104	71		
10			83	88	–34	35	–58	66	–18		–27	31	–34	53	–62	67						
12			67	66	–26	18	–7	16														

Table 3

Calculated and observed F-values of $0kl$ for a Weissenberg photograph of SbOCl. Cu$K\alpha$-radiation.

k	$0k0$		$0k1$		$0k2$		$0k3$		$0k4$		$0k5$		$0k6$		$0k7$		$0k8$		$0k9$	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
0			–10	10	19	18	–23	17	24	19	–93	79	–39	37	4		13		–37	32
1	0		–20	16	–39	38	–1		–57	51	–1		–17	10	37	30	–5		51	34
2	–20	10	–11	11	–62	55	30	22	18	18	13	9	11	10	71	59	15		–16	11
3	0		–1		29	23	51	48	16	11	13	13	19	14	–13	14	–43	38	–42	30
4	12	12	35	40	–22	26	36	23	6	18	–29	26	–37	26	–13		–36	32	–36	28
5	0		–42	50	–12	16	–43	36	–21	22	–43	31	38	25	6	10	25	20	22	21
6	20	23	–75	85	–8	21	–9		67	76	–4		37	42	31	36	22	29		
7	0		–9	19	30	36	66	89	–10	14	16	14	8	14	17	17	–73	63		
8	25	40	49	66	–18	24	25	35	–34	40	–23	22	–62	57	8		–22	27		
9	0		–43	63	–6	14	14	10	–51	59	–47	42	30	34						
10	–29	37	–6		–17	31	–8		1		9		36	27						
11	0		–17	25	50	59	47	51	18	21	–15	21	36	27						
12	46	59	–6		48	48	–30	33	8											
13	0		–45	56	–27	12	–3													

four adjacent cells are given so that the building principle of the structure will be more obvious.

It is seen that antimony and oxygen form a network in a corrugated sheet which is best seen in Fig. 10 where the chlorine atoms have been omitted. Fig. 11 shows projections of the whole structure. Chlorine atoms Cl_1 and Cl_2 are placed in the holes of the antimony-oxygen network and seem to be bound only to one antimony at distances of 2.4_7 and 2.2_9 Å respectively as the shortest Cl–Sb distance after these from Cl_1 and Cl_2 is 3.3_8 Å. We thus have endless sheets of the composition $(Sb_6O_6Cl_4)_\infty$. These sheets seem to be held together by the chlorine Cl_3 which is

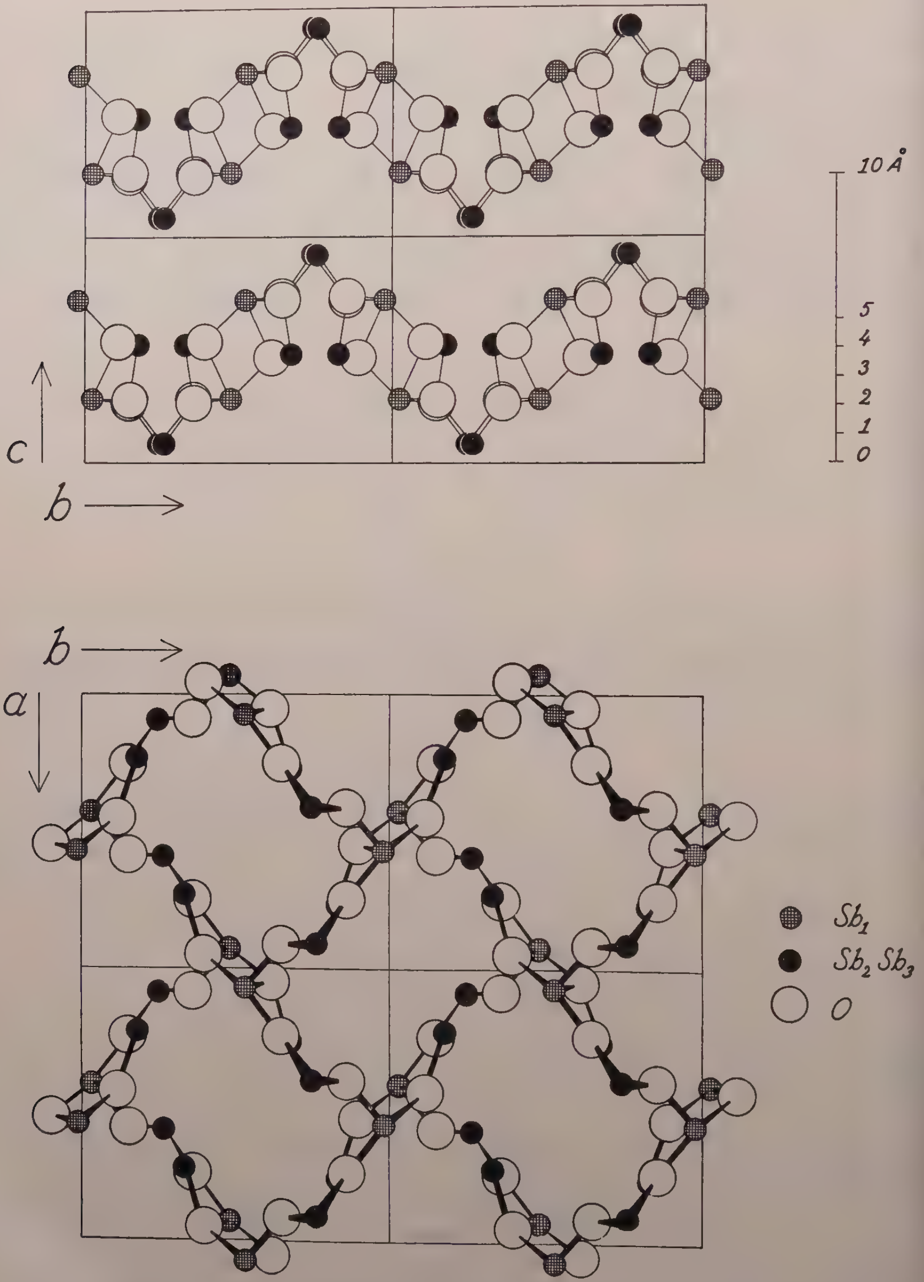
10 Å
5
4
3
2
1
0
c
b
b
a
Sb_1
Sb_2 Sb_3
O

Fig. 10. Projections along the axes, showing the Sb-O network. The Cl-atoms have been omitted. Both projections show four adjacent unit cells.

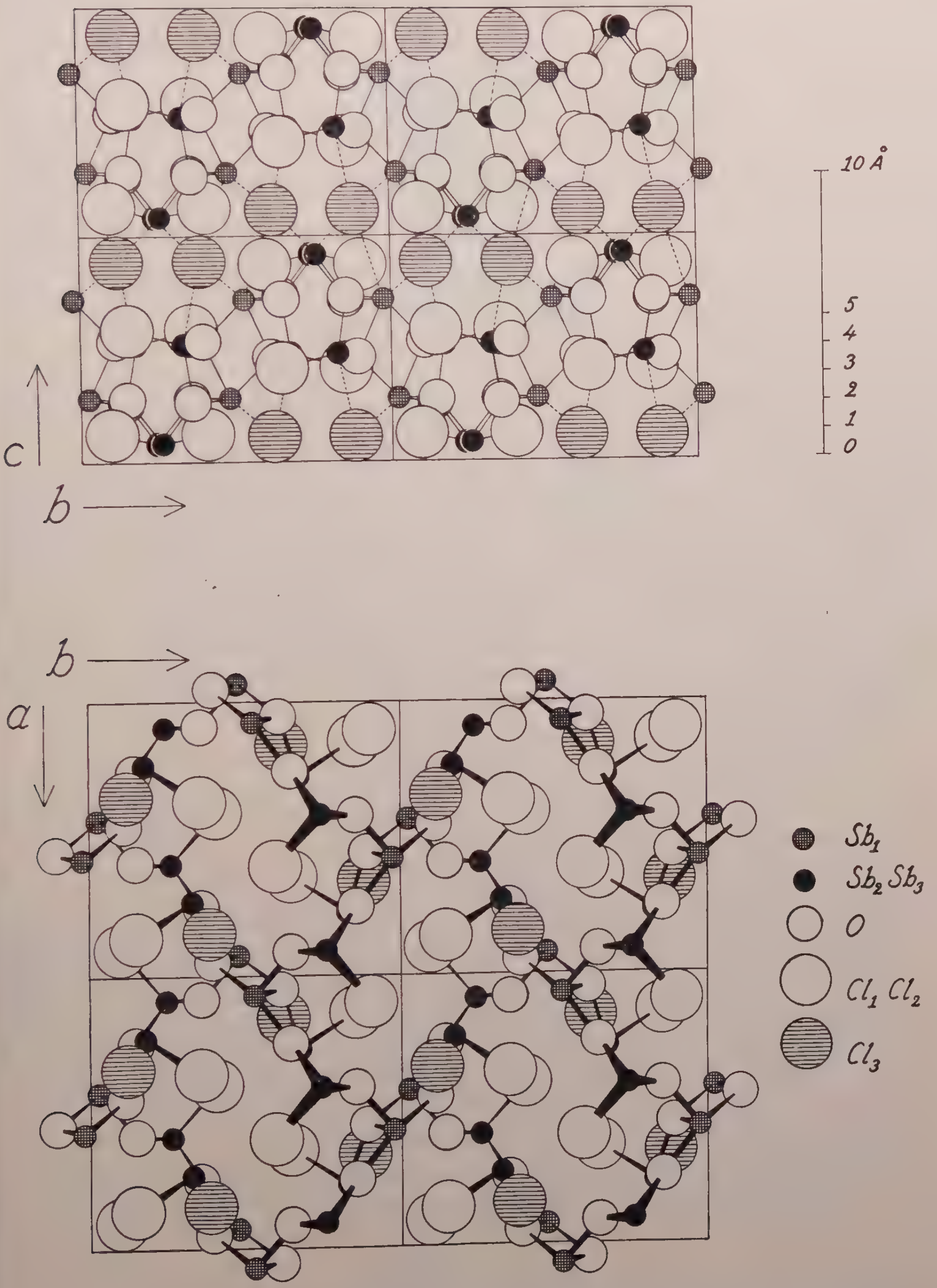
c
b
10 Å
5
4
3
2
1
0
b
a
Sb_1
$Sb_2\ Sb_3$
O
$Cl_1\ Cl_2$
Cl_3

Fig. 11. Projections of the structure of SbOCl along the axes. In each projection four adjacent unit cells.

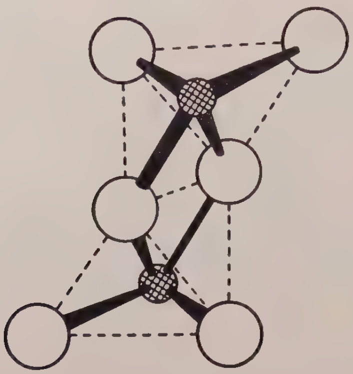

Fig. 12. Oxygen coordination around two antimony of Sb_1. Small circles = Sb_1, large circles = O. Thick lines give Sb-O contacts, and dotted lines O–O contacts.

placed between the sheets. Cl_3 is surrounded by four Sb at the distance 2.9_0, 2.9_1, 3.1_9, and 3.2_0 Å. As mentioned on p. 13 the Sb–Cl distances 2.4_7 ad 2.2_9 are approximately the same as in $SbCl_3$ and the Sb–Cl distances 2.9_0, 2.9_1, 3.1_9, and 3.2_0 are approximately the same as in $Sb_4O_5Cl_2$. The distances together with the coordination number of antimony around the chlorine atoms, which is one for Cl_1 and Cl_2 and four for Cl_3 makes it probable that Cl_1 and Cl_2 are bound with a covalent bond to Sb_2 and Sb_3 respectively, while Cl_3 is mainly in ionic form placed between the endless $(Sb_6O_6Cl_4^{2+})_\infty$ ions. It should be observed that Cl_3 is in close contact with other chlorine and oxygen atoms. Table 4 gives the interatomic distances in SbOCl. (Discussions of the accuracy on p. 14.)

The coordination around Sb_1 is very interesting. It has no chlorine atom nearer than 3.1_9 Å, but is instead surrounded by four oxygen atoms at distances of 1.9_9, 2.1_1, 2.1_7 and 2.1_7 Å as shown in Fig. 12, where a symmetry centre is between the the two antimony atoms. It must be observed that the four oxygens are situated on one side of the antimony atom, although they are not coplanar. In this four coordination the two diagonal O–Sb–O angles are 78° and 134° and the other four O–Sb–O angles are 75°, 97°, 101° and 73°. This coordination figure is very similar to that found around half of the antimony atoms in $Sb_4O_5Cl_2$. It was rather surprising to find it in SbOCl as we have here the same number of the different kinds of atoms.

If in the coordination figures around Sb_2 and Sb_3 respectively we consider only the two oxygens and the chlorine with which they are in close contact and not the chlorine at 2.9 Å and longer distances, we obtain the following angles

around Sb_2: O_2–Sb_2–O_3 87°, O_2–Sb_2–Cl_1 91°, and O_3–Sb_2–Cl_1 97°
around Sb_3: O_1–Sb_3–O_2 73°, O_1–Sb_3–Cl_2 78°, and O_2–Sb_3–Cl_2 98°

All these angles are in a fairly good agreement with angles expected for antimony bound with covalent bonds to three neighbouring atoms.

It thus seems as if the bonds in the sheets $(Sb_6O_6Cl_4^{2+})_\infty$ would be mainly covalent and that there are Cl^--ions between these sheets.

The SbOCl crystals do not seem to have any tendency to form crystal plates. This may be due to the fact that the sheets in the structure are not flat.

A discrete group $Sb_6O_6Cl_4^{2+}$ would be formed if the oxygen O_2 is moved a little so that it comes in close contact only with Sb_1 and Sb_3. As there, however, does

Table 4

Interatomic distances in SbOCl. Å units.

$Sb_1 - 4\ O$	2.1_7	2.1_7	2.1_1	1.9_9	
$Sb_1 - 2\ Cl$	3.1_9	3.2_0			
$Sb_2 - 2\ O$	2.1_1	1.9_9			
$Sb_2 - Cl\ (4\ Cl)$	2.4_7	$(2.9_1$	3.3_8	$3.4_2)$	
$Sb_3 - 2\ O$	2.1_7	2.1_1			
$Sb_3 - Cl\ (2\ Cl)$	2.2_9	(2.9_0)			
$Cl_1 - Sb\ (2\ Sb)$	2.4_7	(3.3_8)			
$Cl_2 - Sb$	2.2_9				
$Cl_3 - 4\ Sb\ (5\ Sb)$	2.9_0	2.9_1	3.1_9	3.2_0	(3.4_2)
$O_1 - 3\ Sb$	2.1_7	2.1_7	2.1_7		
$O_2 - 3\ Sb$	2.1_1	2.1_1	2.1_1		
$O_3 - 2\ Sb$	1.9_9	1.9_9			
$Cl_1 - 4\ O$	3.2_8	3.4_4	3.3_7	3.5_3	
$Cl_2 - 4\ O$	2.8_2	3.1_1	3.3_7	3.5_9	
$Cl_3 - 4\ O$	3.1_9	3.3_2	3.2_4	3.4_2	
$O_1 - 2\ Cl$	2.8_2	3.1_9			
$O_2 - 5\ Cl$	3.2_8	3.4_4	3.3_2	3.3_7	3.5_9
$O_3 - 5\ Cl$	3.1_1	3.3_7	3.5_3	3.2_4	3.4_2
$Sb_1 - 5$ Sb in the same sheet	3.37	3.46	3.52	3.88	3.99
$Sb_1 - Sb_2$ between two sheets	4.15				
$Sb_2 - 3$ Sb in the same sheet	3.50	3.52	3.99		
$Sb_3 - 3$ Sb in the same sheet	3.37	3.50	3.88		
$Cl_1 - 5\ Cl$	3.2_5	3.5_9	3.6_7	3.7_8	3.8_4
$Cl_2 - 3\ Cl$	3.5_2	3.7_8	3.8_4		
$Cl_3 - 4\ Cl$	3.2_5	3.5_9	3.5_2	4.0_0	
$O_1 - 4\ O$	2.5_4	2.6_4	2.6_8	3.1_5	
$O_2 - 3\ O$	2.5_4	2.7_8	3.1_8		
$O_3 - 4\ O$	2.6_8	3.1_5	2.7_8	3.1_8	

not seem to be any special reason to prefer such a configuration to others it was assumed that the proposed structure was more reasonable although the coordination might be somewhat changed by small shifts in the oxygen positions.

As mentioned above the oxygen coordination around Sb_1 is very similar to that around half of the antimony in $Sb_4O_5Cl_2$. In $Sb_4O_5Cl_2$ and $Sb_4O_5Br_2$ we also have antimony oxygen sheets. In these sheets we also have holes although the holes are smaller than in SbOCl. Thus there seem to be some common features in the structures of SbOCl and $Sb_4O_5Cl_2$.

This investigation has been financially supported by Statens naturvetenskapliga forskningsråd. Thanks are due to Professor A. Ölander for laboratory facilities, and to Dr. A. Magnéli of the University of Uppsala for assistance with the Guinier photographs.

SUMMARY

SbOCl has been investigated by X-ray crystallographic methods. It is monoclinic, space group $C_{2h}^5 - P\,2_1/a$ with 12 formula units in the unit cell. Cell dimensions:

$a = 9.52$ kX $= 9.54$ Å
$b = 10.75$ kX $= 10.77$ Å
$c = 7.92$ kX $= 7.94$ Å
$\beta = 103.6°$

with an accuracy of about 0.2 %.

All the atoms are situated in fourfold positions. The atomic coordinates are given on p. 106 and the interatomic distances in table 4. Antimony and chlorine positions have been determined from bounded projections of the electron density. Oxygen positions have been determined from space considerations.

The proposed structure for SbOCl forms a new type. Projections of the structure are given in Figs 10 and 11. Antimony and oxygen form a network of corrugated sheets. Two thirds of the chlorine are bound to these sheets apparently by covalent bonds, while one third is placed between these sheets holding them together apparently by ionic bonds. The composition of the sheets would be $(Sb_6O_6Cl_4^{2+})_\infty$. It is thus remarkable that chlorine is bound in two different ways in this structure, a fact which is indicated both by distances and by coordination.

The oxygen coordination around one third of the antimony atoms (see Fig. 12), seems to be very similar to that found for half of the antimony atoms in $Sb_4O_5Cl_2$.

University of Stockholm Institute of Inorganic and Physical Chemistry May, 1952.

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Tryckt den 8 maj 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

Errata

for the following references:

P. 94, line 5 — read p. 92 for p. 4
P. 95, line 15 — read p. 94 for p. 6
P. 102, line 14 — read p. 100 for p. 12
P. 104, line 6 from below — read p. 102 for p. 14
P. 110, line 2 — read p. 101 for p. 13
P. 110, line 10 — read p. 102 for p. 14

ARKIV FÖR KEMI Band 6 nr 9

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Starch degradation by the α-amylases

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With 2 figures in the text

The most conspicuous feature of the α-amylase action on starch paste is the rapid decrease of the viscosity accompanied by a relatively slow increase of the reducing power. If, for instance, a highly viscous two per cent potato starch paste is broken down by an α-amylase until about ten per cent of the glucosidic linkages have been ruptured, the viscosity is nearly that of water. Already this fact alone shows that the typical, initial action of the α-amylases is a "dextrinization", *i.e.* a breakdown of the long-chain substrate molecules into short-chain dextrins ("α-dextrins"); no high molecular substrate nor degradation products are present after the dextrinization phase of the α-amylase action. The correctness of this view has been shown also in many cases by isolation and fractionation of the α-dextrins (1).

Two types of α-dextrins are formed: a) "normal" α-dextrins in which all linkages between the glucose residues are 1,4-glucosidic linkages, and b) branched α-dextrins containing not only 1,4-linkages but also one, or in exceptional cases possibly more than one, α-glucosidic 1,6-linkage. These dextrins originate from the branched amylopectin component of the starch (1).

After the initial dextrinization a more or less complete "saccharification" of the α-dextrins follows yielding, in all known cases, maltose as the chief product. The normal α-dextrins are completely converted to fermentable sugars; the branched α-dextrins yield sugars and non-fermentable "residual" dextrins. The idea, introduced in 1937 by one of us (2), that the "abnormal" linkages *i.e.* the 1,6-glucosidic linkages of the amylopectin (or glycogen) are the *nuclei* of the residual dextrin molecules, is now generally accepted.

Although many investigators have found distinct differences in the behaviour of different amylases when acting on the substrates (*cf.* literature quoted in (3)) K. H. Meyer and collaborators (4) made the somewhat sweeping statement that all α-amylases (from animal, plant, fungal, or bacterial sources) have the same action and yield ultimately the same products which were supposed to be maltose, glucose and isomaltose in the same proportions in every case. Furthermore it was supposed that the primary products are, with all α-amylases, maltose, maltotriose, and branched dextrins containing three or four glucose units, from which maltose cannot be split off by the rupture of 1,4-linkages, and that glucose which is thought to be formed by all α-amylases alike, is formed from maltotriose and from the branched dextrins with three or four glucose residues.

The scheme proposed by Meyer *et al.* has the merit of simplicity, but is not compatible with results from this and other laboratories. As early as in 1941 it was shown (5) that malt α-amylase, free from maltase, forms considerable amounts of glucose from starch already in the early stages of hydrolysis. The amount of glucose was shown to be about 20 per cent of the amount of maltose formed at the same time.

In the case of salivary amylase, on the other hand, it was shown that little or no glucose was formed under the same conditions. Fermentation experiments showed, for instance, that less than three per cent glucose had been formed when 85 per cent of the starch had been transformed into maltose (fermentation experiments with baker's yeast and with *Torula lactosa*). It may be that small amounts of glucose are formed by salivary amylase in the later stages of hydrolysis (3), but it may be also that some contaminating enzyme is responsible for the glucose formation.

Glucose is formed by malt α-amylase also from amylose (6), but only to a lesser degree from β-amylase limit dextrin (7). This is in agreement with the fact (5) that α-amylase alone yields much more glucose from starch than does a mixture of α- and β-amylase. The explanation is that glucose is formed by malt α-amylase preferably from α-dextrins originating from those parts of the starch that yield maltose with β-amylase: the amylose and the end-chains of the amylopectin, *i.e.* the parts of the starch that are missing in the β limit dextrin.

Myrbäck and Nycander (8) found that carboxylic acids, prepared from α-dextrins by hypoiodite oxidation, are broken down by the amylases. A preparation originating from a normal α-dextrin with six glucose residues on the average ("hexaonic acid") and the corresponding α-dextrin ("hexaose") were compared with starch in this respect. The relative initial velocities are shown in table 1. From this table it appears that the β-amylase attacks all three substrates with the same velocity. On the contrary, the malt α-amylase attacks the high molecular natural substrate with a much higher velocity than it attacks the dextrin with about 6 glucose units; the explanation is the much lower affinity of the enzyme to short chain dextrins (9). The enzyme attacks the corresponding acid at a very low rate, which means that the carboxyl group of the acid impedes the action of the enzyme on adjacent linkages to a considerable degree. The salivary enzyme attacks the dextrin just about as easily as it attacks starch, which means that the affinity of the enzyme to the "hexaose" is not very much lower than that to polysaccharide chains (*cf.* 1). The velocity of the degradation of the acid by salivary amylase has at least the same order of magnitude as the degradation of the dextrin; in the case of salivary amylase the carboxyl group has no such strong action as with malt α-amylase. These observations are important for the understanding of the mechanism of action of the different α-amylases (*vide infra*). It should also be mentioned that malt α-amylase formed 88 per cent maltose and 10 per cent glucose from the dextrin but 51 per cent maltose and *no glucose* from the acid.

Table 1

Intial velocity of the hydrolysis of different substrates by amylases.

	Soluble starch	"Hexaose"	"Hexaonic acid"
β-Amylase	1	0.96	0.92
Malt α-amylase	1	0.17	>0.02
Salivary amylase	1	1	0.57

Salivary amylase gives a higher yield of maltose from starch and α-dextrins of higher molecular weight than does β-amylase. From certain low-molecular α-dextrins just about the same yield of maltose may be obtained by both enzymes (10). In such cases it was found that no glucose was formed by salivary amylase in reasonable times. Small amounts of glucose that were formed after very long times were supposed to arise from the action of contaminating maltase. With malt α-amylase all fractions of α-dextrins yield glucose and maltose, approximately in the ratio 1:5. This glucose formation is certainly an action of the amylase itself (12).

MYRBÄCK and LEISSNER (13), working with mixtures of normal dextrins containing from three glucose units upwards as substrates, were able to conclude that neither salivary amylase nor malt α-amylase nor β-amylase had any action on maltotriose; no glucose at all was formed. It is generally accepted that β-amylase is quite inactive toward maltotriose, and recently ROBERTS and WHELAN (14) have verified our statement that salivary amylase has no action either. In the case of malt α-amylase the evidence is controversary. Certain preparations of this enzyme have been found to hydrolyze maltotriose (15), but since other preparations apparently had no action it must be concluded that pure malt α-amylase does not hydrolyze maltotriose or that the velocity of a hydrolysis is extremely low compared to that of starch.

The non-fermentable limit dextrins formed from starch by Merck's fungal diastase contain, as an average, three glucose units (16). Fractionation experiments show that large amounts of trisaccharides are present together with smaller amounts of disaccharides (presumably isomaltose) and higher saccharides. It is true that at least these higher dextrins are not really *stable* dextrins since they are partly attacked by various α-amylases, but the action is extremely slow and large amounts of trisaccharides are left as limit dextrins. It seems possible to conclude that also in the case of the fungal amylase mentioned certain, if not all, trisaccharides are stable.

Furthermore, we have shown (3) that salivary amylase, acting on starch, produces large amounts of residual dextrins containing, on the average, five glucose units. These dextrins are, to all practical purposes, *stable* dextrins. They are probably not at all attacked by the salivary amylase, and in any case the velocity of their hydrolysis is less than 1/100000 of that of the initial starch hydrolysis. It seems that salivary amylase is not able to release glucose units from most, if not all, of the "primary" limit dextrins with four or five units. This result appears the more likely as the enzyme does not even attack maltotriose (see above). Thus it must be concluded that glucose, maltose, and isomaltose *are not* the only products of starch hydrolysis by the α-amylases. Besides, it should be emphasized that it is by no means proven that glucose and/or isomaltose are formed at all by the enzymatically homogeneous α-amylases. The presence of secondary enzymes in the preparations used does not seem to be excluded. We have tried to illuminate the question of the ultimate products of the enzymatic starch hydrolysis by certain experiments on the action of α-amylases on starch, on a certain α-dextrin fraction, and on the carboxylic acids formed by hypoiodite oxidation of the same dextrin fraction.

Enzymes

a) A partly purified α-amylase preparation from barley malt. It had been freed from β-amylase in the usual way and was purified by acetone precipitation. The α-amylase preparation had no perceptible action on maltose.

b) Purified salivary amylase (3): Sf = 100.

c) A partly purified preparation of fungal amylase. The raw material, "Merck's Diastase", released appreciable amounts of glucose from starch, but was shown to have an action on maltose as well. However, the enzyme acting on maltose could be destroyed by heating of the solution to 56° for 15 minutes. The heated preparations which were used in the following experiments had no perceptible action on maltose in reasonable reaction times.

d) A partly purified β-amylase preparation from soy bean. The preparation did not contain maltase or α-amylase.

Substrates

a) *Starch*: native potato starch.

b) *α-Dextrin*: starting material was an α-dextrin fraction from potato starch, described earlier (17). The fraction (described as fraction IV, tables 1–4 of the quoted paper) was said to contain about 5 per cent glucose and about 15 per cent maltose. The average degree of polymerisation of the nonfermentable part of the preparation was said to be 6.2.

8.95 g of the dextrin preparation (dry weight) was dissolved in 100 ml water and treated with 10 g baker's yeast at 30° until the carbon dioxide evolution had ceased completely. Parallel experiments on a small scale showed that 4.5 per cent glucose and 18 per cent maltose were fermented. (The maltose value may possibly comprise maltotriose.) After fermentation the yeast was removed by filtration through diatomaceous earth. Filtrate and washings were evaporated to nearly dryness, diluted with 100 ml water and evaporated to a final volume of 50 ml. The solution contained 7.02 g substance. Determination of the reducing power before and after complete hydrolysis in *N* sulphuric acid at 100° showed the average degree of polymerisation to be 6.1.

c) *Dextrinic acids*: To 3.6 g of the dextrin mixture mentioned above (free from fermentable sugars) in 40 ml water were added 2 g finely pulverized ionine. 28 ml *N* KOH were then added through a capillary pipette during about 30 minutes. The mixture was agitated mechanically during addition of the alkali and for further 20 minutes. The mixture was acidified with hydroiodic acid and most of the iodine was extracted with chloroform. After removing the remaining iodine by thiosulphate the solution was neutralized with KOH to pH = 6 and poured into four volumes of 99.5 per cent alcohol. After standing over night the yellow precipitate was filtered off and washed with alcohol and ether. The substance was dissolved in water, and traces of iodine were removed by thiosulphate. The yield was 2.5 g. Several other portions were prepared in the same way; yields and properties were the same.

The substance was non-reducing as expected. After complete hydrolysis in *N* sulphuric acid at 100° 100 mg of the substance yielded 88 mg glucose. If the substance is supposed to te, on the average, a hexaonic acid its potassium salt (M = 1044) would correspond to 86 mg glucose.

It should be remembered that the yield of the potassium salt was only about 70 per cent. It seems probable that the acids with higher degree of polymerization have accumulated in the precipitated fraction.

Starch hydrolysis

100 ml 1.89 per cent solution of potato starch was mixed with 70 ml water, 10 ml phosphate buffer solution, and a few drops of toluene. The pH of the buffer solution was 6.9 for salivary amylase, otherwise 5.3. In the experiments with salivary amylase 0.1 g NaCl was added.

Table 2

Hydrolysis of starch by different amalyses.

Time of hydrolysis	Degree of hydrolysis (per cent)			
	Malt α-amylase	Fungal amylase	Salivary amylase	β-amylase
5 min.	4.4	1.9	4.2	2.8
10 ”	6.5	3.9	5.0	4.8
15 ”	9.9	6.0	—	—
20 ”	—	7.3	9.2	11.6
30 ”	13.4	11.9	12.6	16.7
35 ”	—	13.5	—	—
60 ”	16.5	21.3	22.0	20.3
120 ”	18.4	32.3	33.0	24.5
180 ”	19.0	36.1	—	26.7
24 hrs	30.7	40.0	39.8	28.0
120 ”	—	—	—	30.0

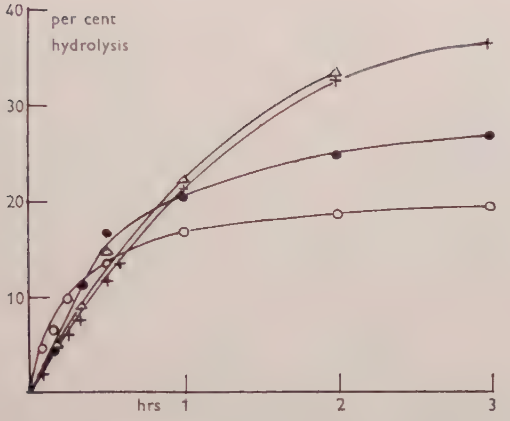

Fig. 1. Starch hydrolysis by different amylases.

○ Malt α-amylase
△ Salivary amylase
+ Fungal α-Amylase
● β-Amylase

Table 3

Formation of fermentable sugars.

Reaction time	Malt α-amylase		Fungal amylase	
	Glucose per cent	Maltose per cent	Glucose per cent	Maltose per cent
60 min.	3.0	13.0	0	43.0
180 ”	6.3	16.4	0	66.8
24 hrs	—	—	0	75.6
48 ”	7.8	48.4	—	—

After addition of 10 ml enzyme solution the increase of the reducing power was followed at 30° (Willstätter-Schudel method). The average degree of hydrolysis was calculated from the reduction values: Table 2.

In Fig. 1 the degree of hydrolysis has been plotted against time. Obviously the α-amylase reactions are widely different. In the first stages of hydrolysis (below 10 per cent) the velocity of the malt α-amylase reaction is nearly twice that of the fungal and salivary amylase reactions. The hydrolysis by the two latter enzymes continues for about 100 minutes at nearly the same rate. After 100 minutes the degree of hydrolysis is about 30 per cent. The malt α-amylase hydrolysis, on the other hand, slows down very strongly when a degree of hydrolysis of about 16 per cent has been reached. This is quite in accordance with results of earlier investigations from this laboratory (1), and the results confirm the previous statement that the mechanism of malt α-amylase action is not the same as that of salivary and fungal amylase. With respect to the form of the hydrolysis curves the two latter enzymes appear similar.

The different action of malt and fungal amylases is shown furthermore by a determination of the fermentable sugars. Glucose and maltose hydrate determinations were carried out according to the fermentation method previously described (17).

In some cases glucose was determined also by fermentation with *Zygosaccharomyces marxianus* that does not attack maltose. All sugar values are calculated as per cent of the theoretical value; 10 or 20 ml of the assay mixtures were used in the fermentation experiments: Table 3.

It appears from table 3 that the fungal amylase used in these experiments does not, or at least not in the earlier stages of the reaction, form glucose whereas in the experiments with malt α-amylase this sugar is formed from the very beginning. It shall not be alleged that glucose may not be formed in the later stages of the fungal amylase reaction, but the experiments prove beyond any doubt that in the range investigated the action of the two enzymes is not at all the same.

Hydrolysis of the α-dextrin

0.7 g dextrin, 5 ml phosphate buffer, enzyme solution, and water to 50 ml. Increase of reducing power determined as above: Table 4.

Since the dextrin yields 86 per cent maltose with β-amylase whereas 41.2 per cent of the glucosidic linkages have been split, it is clear that most of the dextrin molecules were normal, *i.e.* contained no 1,6-linkages. Only those containing ramifications are incompletely saccharified and yield 13.3 per cent limit dextrin of an average degree of polymerization = 6. Thus the limit dextrin has approximately the same average DP as the α-dextrin fraction, but this does not necessarily mean that the

Table 4

Hydrolysis of α-dextrin.

Time of hydrolysis	Degree of hydrolysis (per cent)			
	Malt α-amylase	Fungal amylase	Salivary amylase	β-amylase
0	14.9	14.9	14.9	14.9
5 min.	—	—	17.1	—
30 "	20.7	35.0	20.6	34.9
60 "	21.7	40.2	23.3	36.5
120 "	—	41.6	29.1	37.9
250 "	24.7	—	—	—
24 hrs	38.6	43.5	—	38.6
72 "	—	—	43.5	—
120 "	49.8	—	45.0	41.2
168 "	49.8	50.6	—	—
Glucose, per cent after 120 hrs	—	—	0	0
" 168 "	10.5	5.9	—	—
Maltose, per cent after 120 hrs	—	—	87	86
" 168 "	77	72	—	—
Non-fermentable dextrin, per cent	13	22	15	13
DP	—	—	6	6

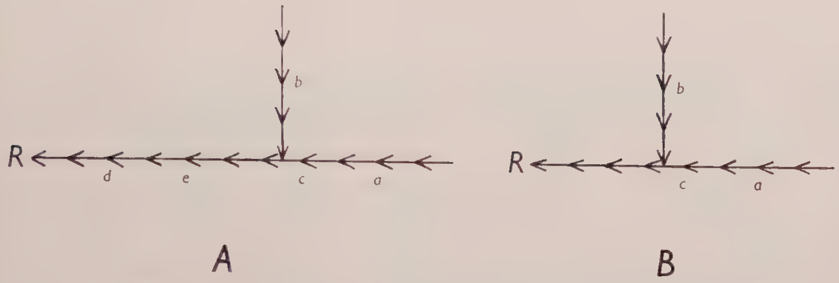

Fig. 2. Branched α-dextrins. *R* = reducing end-group.

amified α-dextrin molecules have not been attacked at all by the β-amylase. They epresent only a small fraction of the original material and may have had a DP igher than the average.

If the branched dextrin molecule, schematically represented in Fig. 2 *A*, is acted pon by β-amylase, it will be split at *a* and *b* and probably at *c*. The reducing end-hain (*R*) will not be ruptured. The original dextrin molecule, containing 14 glucose esidues, will lose six as maltose, and a limit dextrin with eight units will result. n the same way the dextrin 2 *B* will yield a hexasaccharide. It may be assumed that he hydrolysis of 2 *B* together with a large amount of normal dextrin molecules maltohexaose as an average) may represent our experiment with β-amylase.

It is most remarkable that salivary amylase has given about the same result as -amylase. (Table 4.) We have to conclude that salivary amylase does not saccharify he dextrin 2 *B* any further than does β-amylase. This is quite in accordance with ur previous results, which show that salivary amylase, acting on starch (3), yields onsiderable amounts of limit dextrins containing about 5 glucose units per moleule. Obviously the ideas of MEYER and GONON (4) concerning the degradation roducts of starch are not correct, at least not in the case of salivary amylase.

It should be noted that salivary amylase has not yielded glucose from the α-dextrin raction, *i.e.* neither from the normal saccharides nor from the branched dextrins type 2 *B*). It must be concluded that if glucose formation takes place at all its velocity must be extremely small compared to that of the maltose formation.

Contrary to salivary amylase, malt α-amylase forms glucose as a primary product Table 4). The number of linkages split by this enzyme therefore is higher than in he case of salivary amylase and β-amylase, but nevertheless about the same amount f fermentable dextrins is formed in all three cases. Obviously it cannot be assumed hat isomaltose is the only limit dextrin. The same must be said of the fungal enzyme, which produces unfermentable products in even higher yield. Small amounts of gluose were formed by the fungal enzyme. We are, however, not prepared to state hat this glucose formation is due to the amylase proper. Glucose was formed only fter long reaction times and may have been caused by traces of maltase in the enzyme reparation.

Hydrolysis of dextrinic acids

The acids corresponding to α-dextrins are, in general, attacked by the amylases. f the acid originates from a normal α-dextrin, it appears to be saccharified quite ormally; *e.g.* the hexaonic acid yields two moles of maltose and one mole of maltobi-

Table 5

Hydrolysis of dextrinic acids.

Time of hydrolysis	Degree of hydrolysis			
	Malt α-amylase	Fungal amylase	Salivary amylase	β-amylase
0.5 h.	—	—	4.6	—
1	~2	18.4	8.0	16.6
2	~2	18.4	8.4	16.6
24	6.6	19.8	—	18.1
48	—	—	—	19.1
72	—	—	15.3	—
96	17.3	—	—	—
144	—	—	—	21.3
216	—	27.5	—	—
288	20.6	—	—	—
Glucose, per cent	0	<2	0	0
Maltose, per cent	33	37	35	41

onic acid with β-amylase, just as the hexasaccharide gives rise to three moles of maltose If the dextrin and the corresponding acid is branched, the result may be different Consider for instance the dextrin molecule *A* in Fig. 2. When acted upon by β-amylase this dextrin molecule will be split at *a*, *b* and probably at *c*. The reducing end-chain will not be ruptured. An α-amylase, however, will split the molecule at *a*, *b*, *c*, *d* and possibly at *e*. If the reducing group *R* is transformed into a carboxyl group and the acid is acted upon by various amylases, we may expect the following results β-Amylase will most probably rupture the molecule at *a*, *b* and *c*; the yield of maltose will be the same as in the case of the dextrin itself. Likewise an α-amylase will split the molecule at *a*, *b*, and *c*. However, it is an open question if the enzyme will be able to attack the end-chain carrying the carboxyl group (at *d* or *e*). Especially if this chain is short (as in 2 *B*) it seems rather improbable that the enzyme should have any action. Therefore it may well be that the increase of the reducing power caused by the enzyme, may be smaller in the acid than in the reducing dextrin Earlier experiments confirm this view (8). In the following experiments with different amylases 0.316 g potassium salt, 3 ml buffer solution, enzyme, and water to 30 ml was mixed and incubated at 30°. The reducing power was calculated as glucose in per cent of the theoretical yield (Table 5).

All amylases attack the acid and all four, including β-amylase yield about the same amount of maltose. In no case glucose is formed, not even by malt α-amylase which yields considerable amounts of glucose from the corresponding α-dextrin fraction This is in agreement with previous results from this laboratory. It must be concluded that amylases which produce glucose as a primary product (not from maltose) do so by releasing glucose units from the reducing end-chain of the substrate molecules We may add that in our opinion there is only one α-amylase, malt α-amylase, which *with certainty* can be assumed to yield glucose at all.

SUMMARY

Experiments on the hydrolysis of starch, certain α-dextrins, and the corresponding carboxylic ("dextrinic") acids by different amylases allow the conclusion that malt α-amylase forms glucose as a primary product. This glucose is split off from the reducing end-chain of the substrate molecule.

None of the other enzymes investigated has this action. In some cases an extremely slow glucose formation is observed. It may not be an action of the amylases proper and is, in any case, different from that observed with malt α-amylase.

Maltotriose is not hydrolyzed by salivary amylase and probably not by the other α-amylases either.

If isomaltose is formed at all by the α-amylases it is by no means the only stable limit dextrin. All α-amylases and especially salivary amylase produce substantial amounts of limit dextrins which contain 3 or more glucose units and which are at least practically stable against further enzyme action.

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Zur Chemotherapie der Tumoren

Von Hans von Euler und Hans Hasselquist

Die Hemmung einer Tumor-Entwicklung und die Heilung einer bereits bestehenden Krebskrankheit ist, wie schon mehrfach betont wurde, das Hauptziel der chemischen Tumorforschung. Man hat versucht, dieses Ziel zu erreichen durch Stoffe, welche die Tumorzellen *spezifisch* angreifen, und man ist in dieser Bestrebung bestärkt worden durch die epochemachenden therapeutischen Ergebnisse, welche auf dem Gebiet der bakteriellen Infektionen erreicht worden sind (Domagk, Mietzsch, Chain, Waksman, Offe, Lehmann). Die Chemotherapie hat, wie die Strahlentherapie, auch insofern Aussicht auf Erfolg, als die Lebensdauer der Tumorzellen und ihre Stabilität viel geringer, ihre Angreifbarkeit somit viel grösser ist als diejenige der normalen Zellen.

Gleichwohl ist das Aufsuchen spezifischer Tumor-Inhibitoren weder der einzige noch der sicherste Weg zum Ziel. Dem endgültigen, fortschreitenden Wachstum des Krebsgewebes geht ein Kampf zwischen ihm und dem normalen Organismus voraus, in welchem dasjenige Gewebe den Sieg davonträgt, für welches die Bedingungen des Wachstums die günstigsten sind. Zum Rückgang des Krebsgewebes tragen also ausser den Inhibitoren der Cancerzellen auch diejenigen Faktoren bei, welche die Virulenz, die Wachstumsintensität, der im infiltrierten Gewebe vorhandenen normalen Zellen erhöhen. Mit anderen Worten: Wesentlich für den Heilerfolg ist neben der Schädigung der Tumorzellen auch die Verstärkung der Abwehrkräfte des bedrohten Organs.

In welcher Weise es möglich ist, diese Kräftigung des Organismus durchzuführen und den normalen Stoffwechsel quantitativ aufrecht zu erhalten oder zu steigern, wird später besprochen werden.

Zunächst werden hier diejenigen Stoffe behandelt, welche, an ausreichendem Versuchsmaterial geprüft, einen tumorhemmenden Effekt gezeigt haben oder wenigstens vermuten lassen. Wir übergehen also ältere Befunde, aus denen sich im letzten Decennium nichts mehr entwickelt hat, und führen nur diejenigen Ergebnisse an, welche sich zur Beurteilung der hemmenden Stoffe und ihrer Wirkungen theoretisch verwerten lassen und welche für die weitere Forschung richtunggebend sind.

Die bei der Prüfung von Cancer-Hemmstoffen oft erhaltenen widerspruchsvollen Ergebnisse beruhen zum grossen Teil darauf, dass in den verschiedenen Arbeitskreisen Dosen von recht verschiedener Grösse (peroral oder durch Injektion) eingeführt worden sind.

Aromatische Kohlenwasserstoffe

A. Haddow machte 1935 in Verfolgung seiner Arbeiten über Krebserzeugun durch aromatische Kohlenwasserstoffe (vgl. S. 125) an Jensensarkomen tragende Ratten und später an Mäuse-Tumoren wichtige Beobachtungen über den tumorhemmenden Einfluss dieser Substanzen. Am Tage nach der Einimpfung des Sarkoms begann man mit den Injektionen kolloider Lösungen von 15–40 mg Kohlenwasserstoff, die 14–20 Tage fortgesetzt wurden. Starke Hemmungen wurden erziel durch 1,2-Benzpyren und 1,2,5,6-Dibenzanthracen. In 86,5 % von 171 Fällen fan Haddow[1] Hemmungen durch 34 verschiedene Cancerogene, während nichtcancerogene Stoffe zu ca 80 % nicht hemmten. Kleine Dosen einer 0,005 %igen Lösun von Methylcholanthren, intratumoral injiciert, erhöhten das Tumorwachstum.

An Krebspatienten hat K. H. Bauer durch Injektion von Benzpyren in etwa 1/ der Fälle Heilwirkungen, allerdings von begrenzter Dauer, beobachtet.

Zum Vergleich seien einige Daten erwähnt über die kleinsten Mengen cancerogener Kohlenwasserstoffe, mit denen man Krebswachstum *auslösen* kann. Nach Cook genügt bei der Ratte hierzu eine einmalige Injektion von 1 mg 20-Methyl-cholanthren oder Benzpyren. Eine Dosis von 5–25 mg rief mit Sicherheit bei allen angewandten Ratten Sarkome hervor. An einem sehr krebsbereiten Stamm des Stockholmer Vitamin-Institutes konnten E. Klussmann und H. v. Euler durch intramuskulare Injektion von 350 γ 20-Methylcholanthren nach 5 Monaten in 9 von 2 Fällen Tumor hervorrufen. Je kleiner die injizierte Kohlenwasserstoff-Dosis ist desto längere Zeit vergeht bis zum Auftreten der Geschwulst.

In den Mitochondrien werden cancerogene Kohlenwasserstoffe anscheinen elektiv gespeichert (Graffi).

Aliphatische Kohlenwasserstoffe, wie Paraffin und Vaselin rufen nach subcutane und intraperitonealer Injektion an Ratten lokale Sarkome hervor (Druckrey[3] Hamperl). Welche Komponenten in Kunststoffen — ausser Silikaten und Poly amid-Folien — cancerogen wirksam sein sollen ist nicht festgestellt (Druckrey u. D Schmähl, Z. Naturforsch. 7 b, 353; 1952. — Oppenheimer u. Oppenheimer u. Stout Proc. Soc. exp. Biol. Med. 67, 1948).

Aromatische Amine

Dass aromatische Amine die Komponenten bezw. Grundsubstanzen einer grosse Gruppe von Cancerogenen sind, wurde schon früher erwähnt (Arkiv f. Kemi 6, Nr 10; 1953).

β-Naphtylamin soll nach Gahrmann (1934) Blasencarcinom erzeugen.

Grosse Dosen von p-Phenylendiamin können bei geeigneter Diät in Ratten Hepa tome *hervorrufen*. Andererseits wurden nach kleinen Tagesdosen (50 γ, zweima per Woche subcutan injiziert) zuweilen (nicht regelmässig), *Hemmungen* des Wachs tums von Jensensarkomen beobachtet.

p-Phenylendiamin tritt bekanntlich (Miller u. a.) nach peroraler Eingabe vo Dimethylaminoazobenzol (Buttergelb) durch dessen reduktive Spaltung in Organe und im Harn von Ratten auf; ob es als Spaltprodukt des Buttergelbs in der Leber hemmend oder fördernd auf die durch das Buttergelb eingeleitete Hepatombildung wirkt ist noch nicht erwiesen.

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[1] A. Haddow, Proc. Roy. Soc. B, 122, 442; 1937.

[2] J. W. Cook, Chem. Ber. 69, 39; 1936. — J. W. Cook u. E. L. Kennaway, Am. J. Cancer 39, 381 u. 521; 1940. — E. Oberling, M. Guérin u. P. Guérin, Bull. Ass. Franc. Étude de Cance 28, 198; 1939.

[3] H. Druckrey, Dtsch med. Wchschr. 77, 1405; 1952.

Dass *kleine*, peroral oder durch Injektion eingeführte Buttergelbmengen das Auftreten von Jensensarkomen hemmen in Analogie wie dies bei Benzpyren, Methylcholanthren u. a. angenommen wird (Mechanismus des Vorganges noch unbekannt!) ist wenig wahrscheinlich, sofern nicht die gleichzeitig gegebene Diät zu Riboflavin- und Protein-reich, und somit für die Hepatombildung zu ungünstig ist. Denn nach DRUCKREY findet im Rattenorganismus eine Summation der irreversibeln cancerogenen Effekte der Einzeldosen des Buttergelbs statt (siehe u. a. DRUCKREY, Arzneimittelforsch. 1. 383; 1951).

Gleichzeitig mit dem p-Phenylendiamin bilden sich aus Buttergelb in der Leber durch reduktive Spaltung Anilin, p-Phenylendiamin (ev. auch Benzidin und die entsprechenden Methylderivate und Oxydationsprodukte). Reaktionsprodukte des p-Phenylendiamins mit einigen in der Leber vorkommenden Ketonen und Ketosäuren sind früher untersucht und beschrieben worden (EULER, HASSELQUIST u. ERIKSSON, Dieses Arkiv 5, Nr. 48 1953). *Cancerogen* wirken auch die Aminoderivate des Diphenyls und eine Reihe von Azomethiden (Dieses Arkiv 6, Nr 20; 1953).

Im Anschluss an die aromatischen polyzyklischen Amine sind hier die Steroide zu erwähnen, besonders bemerkenswert wegen der von DRUCKREY betonten Beziehungen zwischen cancerogener und östrogener Wirkung.

Als krebshemmende Substanz ist unter den von DODDS u. a. synthetisierten Östrogenen wichtig das *Stilböstrol*, welches das Östradiol an östrogener Wirkung noch übertrifft (CLARKE u. VIETS, J. Am. med. Ass. 121, 499; 1943).

Als aromatisches Anti-cancerogen seien hier noch *Amino-fluoren* und sein Acetylderivat genannt.

Aliphatische und heterozyklische Amine

Unter den tumorhemmenden aliphatischen Amino-derivaten sind in erster Linie die *Urethane* zu nennen. HADDOW und W. A. SEXTON[1] fanden zunächst mit Phenylurethan (Äthyl-phenylcarbamat und Isopropyl-phenylcarbamat), dann auch am Äthylcarbamat selbst eine deutliche Verzögerung des Wachstums von spontanem Mamma-Tumor bei Mäusen, sodann eine ähnliche Verzögerung des Wachstums des Walker-Rattencarcinoms 256, und zwar war der hemmende Effekt des Äthylcarbamates auf das Walker Carcinom noch grösser als derjenige der Phenylcarbamate. Als Wirkungsmechanismus wurde anfangs in Betracht gezogen, dass Urethan mit einem der an der Biosynthese von Nukleotiden beteiligten Amine concurriert. Keine der bisher geäusserten hypothetischen Mechanismen konnte bewiesen werden. Vermutlich hängt die tumorhemmende Wirkung der Urethane zusammen mit ihrem Einfluss auf Leukämie. Wesentlich ist zweifellos die Tatsache, dass Urethane als Mitosengifte fungieren (LETTRÉ[2]).

Amino-Acridin und *Atebrin* sind in kleinen Konzentrationen bezgl. ihrer anticancerösen Wirkung noch unvollständig untersucht.

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[1] A. HADDOW u. W. A. SEXTON, Nature, 157, 500; 1946. — E. PATTERSON, A. HADDOW u. J. M. WATKINSON, Lancet, R. R. PEACOCK u. BECK, Brit. J. exp. Path. 19, 438; 1938.

[2] H. LETTRÉ, Z. Krebsf. 56, 5; 1948. — 56, 297; 1949. — Scientia Pharmaceutica, 20, 75; 1952.

Eine grosse Zahl der hier zu erwähnenden Amine haben sich bereits als Mitosengifte erwiesen; dies gilt, ausser von oben genannten Stoffen von gewissen Gruppen von Kampfgiften, wie z. B. β-Chloräthylamin, ferner vom Adrenochrom, besonders aber vom Colchicin. Wir wollen diese chemisch so verschiedenen Substanzen als Mitosengifte in eine Gruppe zusammenfassen.

Halogen-Verbindungen

Eine grosse Gruppe von tumorhemmenden Halogenverbindungen ist in dem Grade wirksam, in dem Halogen — sei es hydrolytisch, sei es in anderer Weise — abgespalten wird. Dabei dürfen natürlich keine zu grossen Dosen eingegeben werden, da sonst nicht nur die cancerösen, sondern auch die normalen Zellen angegriffen werden.

Ausgehend von Versuchen von Berenblum, Kendall und Orr[1] über die anticancerogenen Wirkungen gewisser Kampfstoffe studierte Crabtree[2] den hemmenden Einfluss organischer Chlorverbindungen auf die Entstehung der durch cancerogene Substanzen erzeugten Tumoren, indem er Mäuse an der gleichen Hautstelle abwechselnd mit Benzpyren und mit der zu untersuchenden Substanz pinselte. Die Latenzzeit des Erscheinens der Tumoren diente als Maas der anti-cancerogenen Wirkung. Der Grad der Hemmung nahm in nachstehender Reihenfolge zu:

Monochlor-acetal, α-Chlordiäthylketon, Methyl-α-Chlorpropylketon, Monochloraceton.

Wurde die Behandlung mit Chloraceton nicht gleichzeitig mit der Benzpyren-Pinselung durchgeführt, sondern erst nachträglich, so zeigte Chloraceton keine Wachstumshemmung, im Gegenteil nahm die cancerogene Wirkung des Benzpyrens zu. Als aber nach der Beendigung der Benzpyren-Pinselung nachträglich Chloraceton gepinselt wurde, traten bei allen Tieren Papillome auf, bei einigen bösartige Epitheliome. Hier superponierte sich offenbar über die Wirkung des Benzpyrens noch ein unbekannter Effekt des Chloracetons.

Tumorhemmungen vermutlich ganz analoger Art erzeugte Dittmar[3] mit Halogenessigsäuren (eingeführt als Salze, Amide oder Ester), besonders aber mit Halogendicarbonsäuren.

Aldehyde, Ketone, aliphatische Dicarbonsäuren

Heptaldehyd aus *Gaultheria procumbens* ruft nach Strong[4] an spontanen Brustdrüsen-Tumoren von Mäusen Erweichung und Rückbildung hervor. Nach Boyland[5] ist jedoch die Wirkung von 50 mg Heptaldehyd auf implantierte Tumoren bei Mäusen gering. Mäuse aus einem Stamm, bei dem Brustdrüsenkrebs häufig und bösartig auftrat, erhielten täglich subcutan Na-Salz von Heptaldehyd-bisulfit Der Grad der dadurch veranlassten Tumorhemmung stieg mit den angewandten Tagesdosen bis zu einem Maximum von 6 mg Aldehyd.

Höher als die Hemmung durch Heptaldehyd ist diejenige durch den *zwei* Doppelbindungen enthaltenden Aldehyd *Citral*. Eine analoge, Cancer-heilende Wirkung des Citrals, wie BOYLAND, beobachtete auch S. OSATO.[1]

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[1] Berenblum, Kendall u. Orr, Biochem. J. 30, 709; 1930.

[2] M. G. Crabtree, J. Path. Bact. 51, 303; 1940. — Canc. Res. 1, 34, 39; 1941.

[3] Dittmar, Z. Krebsforsch. 49, 515; 1940.

[4] Strong, Science 87, 144; 1938.

[5] E. Boyland u. Mawson, Biochem. J. 32, 1892; 1938. — E. Boyland u. Boyland, Biochem J. 33, 618, 1939. — E. Boyland, Biochem. J. 34, 1196, 1940.

Malonsäure hemmt nach BOYLAND bei Mäusen spontane Carcinome und implantierte Sarkome in erheblichem Grade.

Bei Aldehyden sind selten grosse Dosen zur Anwendung gekommen.

Chinone

N. TAKIZAWA[2] wies die cancerogene Wirkung von p-Benzochinon nach, die auftrat, als er die Haut von Mäusen täglich mit 1 %iger Lösung dieses Chinons 200 Tage lang pinselte; es entstanden in 15 20 % der Fälle Papillome, die in Epitheliome übergingen.

KENSLER und Mitarb.[3] machten bald darauf aufmerksam, dass bei p-Phenylendiaminen, welche durch reduktive Spaltung von Buttergelb entstehen, durch Oxydation Farbsalze auftreten, welche mit der cancerogenen Wirkung der Azofarbstoffe zusammenhängen können.

Die gennanten Autoren sahen die freien Radikale als das krebserregende Prinzip an und setzten es mit der hemmenden Wirkung im Hefecarboxylase-System in Beziehung. R. KUHN[4] hat diese Vermutung der amerikanischen Forscher zum Ausgangspunkt einer eingehenderen mit H. BEINERT ausgeführten Untersuchung gemacht. KUHN u. BEINERT fanden, in Bestätigung der betr. Resultate von KENSLER u. Mitarb. (l. c.), dass die WURSTER'schen Farbsalze und — noch mehr — höhere Oxydationsprodukte der Diamine starke Cancerogene sind[5] und wiesen die besonders starke Hemmung des p-Benzochinons auf die Hefencarboxylase nach; sie halten demgemäss das p-Chinon für den eigentlichen Tumor-Erreger, und betonen, dass die protrahierte Chinon-Wirkung im Organismus vermutlich stärker ist als die der Injektionen nach N. TAKIZAWA.

Versuche mit peroraler und subcutaner Eingabe von Naphtazarin, Benzochinon und Naphtochinon sind in diesem Institut im Gang, und zwar besonders mit kleinen Chinonmengen, um ev. *Tumorhemmungen* nachzuweisen.

Dass Cystein die Chinonwirkung hemmt wurde bereits von KENSLER, YOUNG und RHOADS sowie von KUHN und BEINERT erwähnt. MCCARTY u. AVERY[6] berichten über den Einfluss der SH-Gruppe im System Chinon-Desoxyribonukleinsäure[7].

Nach Adrenalin (Adrenochrom)-Injektionen konstatierten EULER u. MALMBERG sowie EULER, RÖNNESTAM-SÄBERG u. I. PETTERSSON[8] Rückgang von Jensensarkomen bei Ratten[9].

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[1] S. OSATO, Gann, 39, 159; 1951.

[2] N. TAKIZAWA, Proc. Imp. Acad. Tokyo 16, 309; 1940.

[3] C. J. KENSLER, C. O. DEXTER u. C. P. RHOADS, Cancer Res., 2, 1; 1942. — C. J. KENSLER, N. F. YOUNG u. C. P. RHOADS, J. biol. Chem. 143, 465; 1942.

[4] R. KUHN und H. BEINERT, Chem. Ber. 76, 904; 1943. — Chinon als Hemmstoff der Carboxylase: R. KUHN, Chem. Ber. 80, 2; 1947.

[5] Bemerkenswert ist auch die Beobachtung von H. S. REED über die Blockierung der Mitosen durch Chinone in jungen Pflanzengeweben (Experientia 5, 237; 1949). Siehe auch die Zusammenfassung von HOFFMANN-OSTENHOF, Experientia 3, 137 u. 176; 1947.

[6] MCCARTY u. AVERY, J. exp. Med. 81, 501; 1945. — AVERY, J. exp. Med. 79, 137; 1944.

[7] Auch die antitumorale Wirkung mancher ungesättigter Ketone (Chalkone) wird durch Cystein inaktiviert. (Siehe hierzu auch Z. f. Naturforsch. 4 b 276; 1944.)

[8] EULER, I. RÖNNESTAM-SÄBERG u. I. PETTERSSON, Arkiv f. Kemi etc. 20 A, Nr. 12; 1944/45. Siehe weiter im Abschnitt Mitosen-Hemmung.

[9] Vgl. H. LETTRÉ, Ergebn. d. Enzymforsch. 10, 269; 1949. — Angew. Chem. 63, 421; 1951. — Z. physiol. Chem. 291, 99; 1952.

Manche cancerogenen Kohlenwasserstoffe und andere Cancerogene gehen im Organismus in Oxydationsprodukte über und bilden auch Chinone, unter diesen kommen auch *tumorhemmende* Stoffe vor. Die von Boyland[1] untersuchte Oxydation zu Chinonen lässt sich nach Cook und Martin[2] zur Deutung ihrer cancerogenen Wirkung nicht verwerten[3]. Nachdem Chalmers und Crowfoot[4] nach Eingabe von Benzpyren in Ratten und Mäuse eine Substanz isolieren konnten, die sich bald als 8-Hydroxy-3,4-benzpyren erwies, haben sich englische und amerikanische Forscher[5] mit diesem Oxydationsprodukt beschäftigt und haben die Bildung von Chinonen nachgewiesen.

Schwefel-haltige Hemmungsstoffe

Sulfhydryl-Verbindungen greifen durch ihre hohe Reaktionsfähigkeit, besonders gegenüber Carbonylgruppen, weitgehend sowohl in die cancerogenen als in die antitumoralen Vorgänge ein.

Auch die tumorhemmenden Wirkungen gewisser Kohlenwasserstoffe werden nach den älteren Befunden von J. White und A. White[6] durch Stoffe wie Cystin und Glutathion aufgehoben.

Schwefelhaltige Kampfstoffe wie auch manche schwefelhaltigen Glukoside sind als Enzymgifte erkannt worden.

Im Arbeitskreis des Verf. wurden schon vor längerer Zeit Versuche über die Wirkung von Knoblauch-Extrakten auf die Entwicklung von Tumoren angestellt, an dieser Wirkung sind ja S-haltige Stoffe beteiligt.

Was besonders die Wirkungen des *Alliins* betrifft, der von A. Stoll[7] aus Knoblauch zuerst in reinem Zustand gewonnenen und strukturell aufgeklärten Substanz, so hatten die von Euler und Lindeman[8] mitgeteilten täglichen Änderungen der Sarkomgewichte nach 1–2-maliger subcutaner Injektion von 5–10 mg Alliin keinen Rückgang der Sarkome hervorgerufen. Eine folgende Versuchsserie[9] hatte nach intratumoraler Injektion von 1–3 mg Alliin in 2–4 ccm grosse Sarkome sowie nach einmaliger intramuskularer Injektion in 11 Fällen von 20 einen Rückgang bezw. Verschwinden des Tumors erzeugt (Einnahme von Alliin durch Trinkwasser, etwa 5 mg per Tag und Ratte), ergab keine toxischen Wirkungen, es zeigte sich eine erhöhte Wachstumszunahme der wachsenden Ratten.

Eine neuerdings von O. Klamerth angestellte dritte Versuchsreihe (30 mg Alliin subcutan auf 10 Tage verteilt) rief keine Hemmung der Entwicklung von Jensensarkomen hervor. Alliin scheint weder cancerhemmend noch cancerogen zu sein.

Dagegen ergab eine, allerdings kurze, Versuchsreihe mit *Vollextrakten von schweizerischem Knoblauch* (das Material war von Herrn Direktor Prof. Dr. A. Stoll freundlichst zur Verfügung gestellt) in etwa 1/4 der Fälle Rückgang der Sarkome (noch unpublizierte Versuche von O. Klamerth).

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[1] E. Boyland, Biochem. J. 27, 791; 1933.

[2] Cook u. Martin, Soc. 1940, 1125.

[3] Chinone spielen auch als Abbauprodukte von cancerogenen Azofarbstoffen eine vermutlich wichtige Rolle; vgl. S. 127.

[4] J. G. Chalmers u. D. Crowfoot, Biochem. J. 35, 1270; 1941.

[5] I. Berenblum, D. Crowfoot, Holyday u. Schoental, Cancer Res. 3, 151; 1943.

[6] J. White u. A. White, Proc. Soc. exp. Biol. Med. 39, 527; 1938.

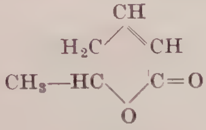
[7] A. Stoll u. E. Seebeck, Helv. Chim. Acta 31, 189; 1948. — A. Stoll u. Mitarb. Helv. Chim. Acta.

[8] Euler u. G. Lindeman, Dieses Arkiv 1, Nr. 12; 1948.

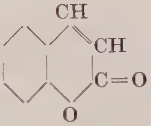
[9] Euler, Kem. Arbeten. Ny Följd B 12; 1949.

Spezifische Hemmungsstoffe

Kuhn und Moewus[1], sowie G. M. und R. Robinson[2] haben aus Vogelbeeren und aus Malz Cumarin-Derivate isoliert, gegen welche Fibroplasten besonders empfindlich sind. Diese Cumarine hemmen die Keimung von Samen und Pollen, das Wachstum gewisser Mikroorganismen und gewisser tierischer Gewebe (nicht der Carcinome). Es handelt sich um Parasorbinsäure und Derivate derselben. Sehr wirksam erwies sich *Cumarin*. Über die Art der Wirkung dieser Blastocholine ist noch nichts näheres bekannt.



I Parasorbinsäure



II Cumarin

Ein von Euler[3] aus schwarzen Johannisbeeren und Citrusfrüchten isolierter Stoff ist vermutlich mit diesen identisch oder nahe verwandt.

Nukleinsäuren. — Pteroyl-Derivate

Nukleinsäuren und Nukleoproteide und ihr Stoffwechsel stehen zum Wachstum sowohl der normalen wie der Tumorzellen in nächster Beziehung.

In tumortragenden Tieren riefen Adenylsäure und Guanylsäure Hemmungen des Tumorwachstums hervor. Dagegen *förderte* Uridylsäure das Wachstum; Cytidylsäure hatte wenig Effekt (Chargaff[4]; Parsons u. Gulland[5]).

Lewisohn u. Mitarb.[6] beobachteten vollständigen Rückgang bei 11 von 28 Mäusen nach Eingabe von Pteroyl-triglutaminsäure. Nach Spies ist diese Substanz beim Menschen aber nicht wirksam. Teropterin soll Mamma-tumoren bei Mäusen hemmen, nicht bei Menschen. Diopterin zeigte keinen sicheren Effekt.

Die Einwirkung von Adenosinphosphorsäure, Adenosintriphosphorsäure, Ribonukleinsäure und Desoxyribonukleinsäure sowie von Nukleoproteiden wird in Zusammenhang mit anderen den Tumor-Stoffwechsel beeinflussenden Substanzen besprochen.

Einfluss der Proteine, Nukleoproteide und Nukleinsäuren auf die Ausbildung der Hepatome durch Buttergelb

Hier sind zunächst die Angaben amerikanischer Forscher[7] über die Verzögerung der Hepatombildung durch einen hohen Proteingehalt der Nahrung von Ratten zu erwähnen, Angaben die auch mit unseren Befunden in Übereinstimmung stehen. Es handelt sich hier zum Teil um unspezifische, zum Teil um spezifische Protein-

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[1] R. Kuhn, D. Jerschel, F. Moewus und E. F. Möller, Naturwiss. 31, 468; 1943.

[2] Medawer, G. M. Robinson u. R. Robinson, Nature 151, 195; 1943.

[3] Euler, H. Söder u. M. Malmberg, Z. f. Hygiene u. Inf. Krankh. 116, 672; 1935. — Euler, Kem. Arbeten, Ny Följd B, Bd 13, II; 1950.

[4] Chargaff, Ann. Rev. Biochem. 17, 211; 1948.

[5] Parsons u. Gulland, Symp. Soc. exp. Biol. 1, 179; 1947.

[6] Lewisohn *et al.* Science, 104, 436; 1946.

[7] Siehe z. B. E. C. Miller, C. A. Baumann, H. P. Rusch, Cancer Res. 5, 713; 1945.

und Nukleoproteid-Wirkungen, z. B. um diejenige eines Proteid-Komplexes *Avidin.*[1] Tumoren sollen anormal hohen Biotin-Zufuhr brauchen und durch Biotin-Mangel gehemmt werden. Dies steht in Übereinstimmung mit der Beobachtung, dass der Zusatz von Biotin zur Diät gegen die Ausbildung von Hepatomen durch Azofarbstoffe schützt.[2]

Wie das Wachstum der Tumoren, so ist auch ihr Abbau und Rückgang in vivo beherrscht von zwei entgegengesetzt wirkenden Gruppen von Faktoren,

den cancerogenen Stoffen und

den am normalen Stoffwechsel beteiligten Vitaminen und Hormonen.

Keine dieser beiden biologischen Stoff-Klassen ist hinsichtlich ihres Wirkungs-Mechanismus vollständig erkannt. Immerhin deuten viele Tatsachen darauf hin, dass beide an den Enzymen des Stoffwechsels, und zwar oft an den Enzymen des oxydo-reduktiven Stoffwechsels angreifen. (Vgl. EULER, Atti della Società Italiana di Patologia, Vol. II 1951. Vortrag in *Turin.*)

Besonders deutlich tritt dieser Antagonismus hervor bei denjenigen Versuchen, bei welchen gleichzeitig zwei Cancerogene bezw. zwei Anti-cancerogene zur Anwendung gekommen sind. Dieselben werden in einer folgenden Mitteilung zusammenfassend behandelt.

Vom biochemischen Standpunkt aus und in Zusammenhang mit dem vorliegenden Abschnitt über den Einfluss von Proteinen und Proteiden auf die Vorgänge am Buttergelb bei der Hepatombildung in der Leber, sind in erster Linie die Fragen experimentell zu beantworten: Welcher Anteil einer gewissen in der Zeit t peritoneal eingegebenen Menge Buttergelb gelangt in die Leber[3], wie gross ist der in der Leber an Eiweiss adsorbierte Anteil, und welche Spalt- und Umwandlungsprodukte des Buttergelbs nehmen an der cancerogenen Wirkung Teil?

Messungen

Zu den Messungen, die in einer folgenden Arbeit mitgeteilt werden sollen, haben wir uns folgender Methoden bedient:

Buttergelb-Bestimmung: Colorimetrie nach Zusatz von HCl und Bestimmung des Extinktion.

Bestimmung des p-Phenylendiamins: a) Colorimetrie nach Zusatz von Redukton, b) Farbenreaktion durch Fichtenspan.

Fraktionierung der Spaltprodukte: Papierchromatographie und Bestimmung der R-Werte durch fluorometrische Beobachtung.

Konzentrations-Grenze der Beobachtung

Konzentr. des p-Phenylendiamins/ml	Reaktion mit Redukton + HCl	Reaktion mit Fichtenspan
100 γ	Gelb	Stark rotbraun
10 γ	Gelb	Rotbraun
1 γ	Sehr schwach	Schwach rotbraun
0,1 γ	Unsichtbar	Unsichtbar

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[1] LAURENCE, Science, 94, 88; 1941.

[2] So erklärt sich auch, dass transplantierte Hepatome nach Biotineingabe in höherem Grad zurückgebildet werden.

[3] Die Wichtigkeit in dieser Hinsicht zu quantitativen Einsichten zu gelangen, haben auch H. DRUCKREY und K. KÜPFMÜLLER in einem bemerkenswerten Beitrag zur theoretischen Pharmakologie „Dosis und Wirkung" (Die Pharmazie, 1. Ergänzungsbd. 8. Beih. 1949) betont.

Die papierchromatographische Untersuchung wurde an folgender Lösung ausgeführt: Das Organ wurde mit 96 %igem Alkohol verrieben; die ungelösten Reste wurden durch Filtration entfernt, die alkoholische Lösung auf ein geringes Volumen eingeengt. Sämtliche Leberpräparate wurden auch hinsichtlich ihres Spaltungsgrades und ev. anwesenden freien Buttergelbs untersucht.

Ratte	Organ	Fluoreszenz im UV	R_f-Wert des Fluoreszenz-Fleckens im Chromatogram
45; behand. mit Butterg.	Leber	Gelbgrün	0
	Pankreas	Schwach blauviolett	0
38; Buttergelb; Hepatom	Leber	Gelbgrün	Violetter Flecken 0.97
	Milz	Schwach blauviolett	0
	Nieren		—
39; Buttergelb; »	Leber	Gelbgrün	0
	Milz	Schwach blauviolett	—
20; Buttergelb; »	Leber	Gelbgrün	0
	Lunge	Schwach blau	—
345; Jensensark. behandelt mit Knoblauchextrakt	Leber	Gelbgrün	0
	Sarkom	Hellblau	—
Normal behand. mit 26 mg Hydrazobenzol	Leber	Gelbgrün	0
Die Harnprobe an dieser Ratte gab keine Reaktion auf ev. Spaltprodukte.			

a) *Bearbeitung normaler Rattenlebern*

Das Material wurde im Mörser mit 96 %igem Alkohol fein verrieben, das Ungelöste wurde abfiltriert und getrocknet (Trockenpräparat gesammelt). Die alkoholische Lösung wurde im Vacuum bei 40° auf 5 ml eingeengt, wobei sich Fett abschied und abzentrifugiert wurde. Die klare Lösung wurde zu den Fluoreszenz-Proben und zum Chromatogramm verwendet.

b) *Bearbeitung normaler Rattenlebern nach Inkubation mit Buttergelb*

Normalleber der Ratte Nr. 316 wurde im Mörser mit einer Suspension von 1 g Buttergelb in 2 ml Wasser verrieben. Dieser Brei stand bei 37° 18 Stunden lang, wurde dann zentrifugiert und die Lösung auf p-Phenylendiamin geprüft, teils durch Zusatz von Redukton, teils mit Holzspänen, und zwar sowohl direkt als nach Zusatz von wenig 4-n. HCl. Das Centrifugat wurde mit 10 ml Alkohol aufgeschlämmt, wobei unverändertes Buttergelb erhalten wurde. Nach Auswaschen mit Alkohol wurde mit 2-n. Alkalilauge 30 Minuten aufgekocht, mit HCl angesäuert und wieder auf die Anwesenheit von p-Phenylendiamin geprüft. Es wurden keine mit unseren Methoden nachweisbaren Mengen dieses Diamins gefunden; jedenfalls betrug die im Brei enthaltene Menge p-Phenylendiamin weniger als 1 γ.

Parallelversuch mit normaler Leber (Ratte 21.XI. 52) ergab das gleiche Resultat.

Da in Betracht gezogen wurde, dass das durch reduktive Spaltung in der Leber unserer lebenden Buttergelb-Versuchstiere entstandene p-Phenylendiamin zu Beginn der Hepatom-Bildung in der Leber oxydiert wird, haben wir folgende zweite Versuchsreihe angestellt:

II a. Normalleber, wie oben verrieben, mit einer Suspension von 1 mg Buttergelb in 5 ml *einer 1 %igen* H_2O_2*-Lösung*. Inkubation bei 37° während 18 Stunden. Aufarbeitung wie unter I a.

II b. Normalleber der Ratte BDI 3775, behandelt wie unter II a.

In beiden Versuchen war die p-Phenylendiamin-Menge geringer als 1 γ.

Weitere Versuche müssen zeigen, ob ausser p-Aminophenol und p-Benzochinon noch andere Oxydationsprodukte des reduktiv gespaltenen Buttergelbs nachgewiesen werden können.

Stockholm, Universität, Vitamin-Institut und Inst. f. organisch-chemische Forschung.

Tryckt den 8 maj 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 11

Communicated 14 January 1953 by A. WESTGREN and G. HÄGG

The crystal structures of $(Mo,W)_{10}O_{29}$ and $(Mo,W)_{11}O_{32}$

By BIRGITTA BLOMBERG, LARS KIHLBORG, and ARNE MAGNÉLI

With 1 figure in the text

Introduction

The remarkable relationship existing between the crystal structures of Mo_8O_{23}, Mo_9O_{26}, and $W_{20}O_{58}$ (1, 2) has led us to carry out geometrical considerations of various atomic arrangements of analogous structural type (3) and to undertake investigations of mixed molybdenum tungsten oxides of compositions close to those of the above single metal oxides. *viz.* $MeO_{2.9}$. In the course of the experimental studies several phases have been found. Structure determinations have so far been carried out for two of the double oxides and will be described here. They have shown that the lattices of these substances are of the same type as those of Mo_8O_{23} and Mo_9O_{26} but correspond to the formulae $(Mo,W)_{10}O_{29}$ and $(Mo,W)_{11}O_{32}$. The number of known members of the "homologous series" Me_nO_{3n-1} (3) has thus increased to four.

Experimental

By heating a mixture of molybdenum, molybdenum trioxide and tungsten trioxide of composition $Mo_{0.85}W_{0.15}O_{2.9}$ in an evacuated, sealed silica tube at 750° C for 50 hours small, dark blue crystal needles were formed (substance *A*). A small amount of dark green, rather long and extremely thin, needle-shaped crystals (substance *B*) were found in a preparation obtained by heating an equimolecular mixture of molybdenum trioxide and tungsten trioxide in an evacuated, sealed silica tube at about 700° C for 45 days, where it appeared together with a major constituent of a micro-crystalline, yellowish green product of the approximate composition $MoWO_6$ (4).[1]

Rotation and Weissenberg photographs of the two crystal species were taken with rotation around the needle axes using Cu-*K* radiation. The intensities of the reflections were estimated visually and the intensity scales of the layer lines were correlated by means of "Weissenberg oscillation photographs" (1). The Fourier summations were generally carried out by means of the HÄGG-LAURENT machine (5).

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[1] The cause of the formation of the reduced substance *B* is the instability of molybdenum trioxide, when heated *in vacuo* (unpublished experiments).

The crystal structure of $(\mathbf{Mo,W})_{10}\mathbf{O}_{29}$[1]

The Laue symmetry of substance A was found to be $2/m$ and the missing spectra were those characteristic of the space-groups $P2/a$ and Pa. Reflections $0k0$ were not registered in the single crystal photographs as the extension of the crystal needles was in the direction of the monoclinic axis but the occurrence of the reflection 010 in the powder photograph exluded the symmetry $P\,2_1/a$. The unit cell dimensions as obtained from the single crystal photographs were

$$a = 17.0\text{ Å},\ b = 4.00\text{ Å},\ c = 17.5\text{ Å},\ \beta = 111°,$$

these values being in agreement with those derived for the hypothetic structure $Me_{10}O_{29}$ (3). Structure factors for reflections $h0l$ calculated on the basis of the metal atom positions of this ideal lattice of symmetry $P\,2/a$ were also in accordance with the observed data and thus allowed of the determination of the signs of most reflections. The x and z co-ordinates of the metal atom positions were refined by calculation of the projection of the electron density function $\varrho(xz)$, which suggested a statistical distribution of the molybdenum and tungsten atoms.

As no reflections hkl with k larger than 2 were registered the y parameters could not be obtained with a very high degree of accuracy. Assuming all the metal atoms to be situated in two planes $y = \frac{1}{2} \pm \nu$ as in Mo_8O_{23} and Mo_9O_{26} the ratio

$$\sum_h \sum_l |\mathrm{F}(h0l| : \sum_h \sum_l |\mathrm{F}(h1l)| : \sum_h \sum_l |\mathrm{F}(h2l)| = 1 : \cos\nu : \cos 2\nu \quad (h = \text{even})$$

led to a value of ν of the same magnitude as in the molybdenum oxides, *viz.* ~0.08. A distribution of the metal atoms on $y = 0.42$ and $y = 0.58$ in a way analogous to that present in the puckered molybdenum atom layers of Mo_8O_{23} and Mo_9O_{26} gave fair agreement between observed and calculated values of structure factors $\mathrm{F}(h1l)$ and $\mathrm{F}(h2l)$.

No reliable conclusions could be drawn from the electron density maps concerning the positions of the oxygen atoms, which thus had to be derived from spatial and functional considerations. In this way an oxygen atom arrangement was arrived at, consistent with that predicted for the hypthetic structure $Me_{10}O_{29}$ and throughout corresponding to appropriate interatomic distances.

The crystal structure thus derived is illustrated in Fig. 1 a and the atomic arrangement is given below.

Cell content: 2 $(Mo,W)_{10}O_{29}$.
Space group: $P\,2/a$.

4 Me_1 in 4 (g):	0.117	0.42	0.050	2 O_6 in 2 (c):	0	$\frac{1}{2}$	0
4 Me_2 in 4 (g):	0.353	0.58	0.150	4 O_7 in 4 (g):	0.365	0.50	0.050
4 Me_3 in 4 (g):	0.091	0.58	0.252	4 O_8 in 4 (g):	0.235	0.50	0.100
4 Me_4 in 4 (g):	0.325	0.42	0.352	4 O_9 in 4 (g):	0.105	0.50	0.151
4 Me_5 in 4 (g):	0.065	0.58	0.457	4 O_{10} in 4 (g):	0.472	0.50	0.201
4 O_1 in 4 (g):	0.117	0.00	0.050	4 O_{11} in 4 (g):	0.340	0.50	0.251
4 O_2 in 4 (g):	0.353	0.00	0.150	4 O_{12} in 4 (g):	0.208	0.50	0.302
4 O_3 in 4 (g):	0.091	0.00	0.252	4 O_{13} in 4 (g):	0.078	0.50	0.354
4 O_4 in 4 (g):	0.325	0.00	0.352	4 O_{14} in 4 (g):	0.440	0.50	0.430
4 O_5 in 4 (g):	0.065	0.00	0.457	4 O_{15} in 4 (g):	0.315	0.50	0.465

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[1] The structure determination was carried out by B. B. and A. M.

It may be that the oxygen atoms O_1–O_5 are somewhat displaced from the plane $y = 0$ to form puckered layers conformable to the metal atom layers in the way previously suggested for the corresponding structural elements in Mo_8O_{23} and Mo_9O_{26} (1).

The crystal structure of $(Mo,W)_{11}O_{32}$[1]

The dimensions of the monoclinic unit cell of substance *B* were found to be

$$a = 16.6\ \text{Å},\ b = 4.00\ \text{Å},\ c = 18.7\ \text{Å},\ \beta = 74°$$

and thus in agreement with those of the hypothetic structure $Me_{11}O_{32}$ (3). The X-ray diffraction pattern showed reflections only in the close vicinity of the reciprocal lattice points of the tetragonally modified basic ReO_3-type sublattice. The signs of the structure factors $F(h0l)$ were thus assumed to be the same as those of the adjacent sublattice reflections in conformity with the method utilized when determining the crystal structure of $W_{20}O_{58}$ (2).

The electron density map $\varrho(xz)$ showed maxima on two interpenetrating systems of square networks, mutually related by the centrosymmetry of the unit mesh, and could be interpreted as containing real maxima corresponding to the metal atom sites of the hypothetic structure $Me_{11}O_{32}$ and diffraction peaks originating from the great number of weak, not detectable reflections being omitted in the Fourier series (6). Due to the diffraction effects no definite conclusions could be drawn concerning the distribution of the molybdenum and tungsten atoms on the various positions.

The y co-ordinates of the metal atoms and the oxygen atom positions were determined in the same way as for $(Mo,W)_{10}O_{29}$. The atomic arrangement thus derived (*cf.* Fig. 1 b) is consistent with the hypothetic structure $Me_{11}O_{32}$ and shows reasonable interatomic distances and fair agreement between calculated and observed structure factor values. The structure may be described in the following way:

Cell content: 2 $(Mo,W)_{11}O_{32}$.
Space-group: $P\,2/a$.

2 Me_1 in 2 (*e*):	$\frac{1}{4}$	0.60	0
4 Me_2 in 4 (*g*):	0.418	0.40	0.092
4 Me_3 in 4 (*g*):	0.088	0.40	0.183
4 Me_4 in 4 (*g*):	0.257	0.60	0.277
4 Me_5 in 4 (*g*):	0.427	0.40	0.370
4 Me_6 in 4 (*g*):	0.096	0.60	0.458
2 O_1 in 2 (*e*):	$\frac{1}{4}$	0.00	0
4 O_2 in 4 (*g*):	0.418	0.00	0.092
4 O_3 in 4 (*g*):	0.088	0.00	0.183
4 O_4 in 4 (*g*):	0.257	0.00	0.277
4 O_5 in 4 (*g*):	0.427	0.00	0.370
4 O_6 in 4 (*g*):	0.096	0.00	0.458
2 O_7 in 2 (*c*):	0	$\frac{1}{2}$	0
4 O_8 in 4 (*g*):	0.334	0.50	0.046
4 O_9 in 4 (*g*):	0.169	0.50	0.091
4 O_{10} in 4 (*g*):	0.003	0.50	0.137
4 O_{11} in 4 (*g*):	0.337	0.50	0.185
4 O_{12} in 4 (*g*):	0.173	0.50	0.230
4 O_{13} in 4 (*g*):	0.008	0.50	0.276
4 O_{14} in 4 (*g*):	0.342	0.50	0.324
4 O_{15} in 4 (*g*):	0.177	0.50	0.367
4 O_{16} in 4 (*g*):	0.496	0.50	0.432
4 O_{17} in 4 (*g*):	0.333	0.50	0.467

Even in this substance the oxygen atoms O_1–O_6 may be somewhat displaced from the plane $y = 0$ to form puckered layers.

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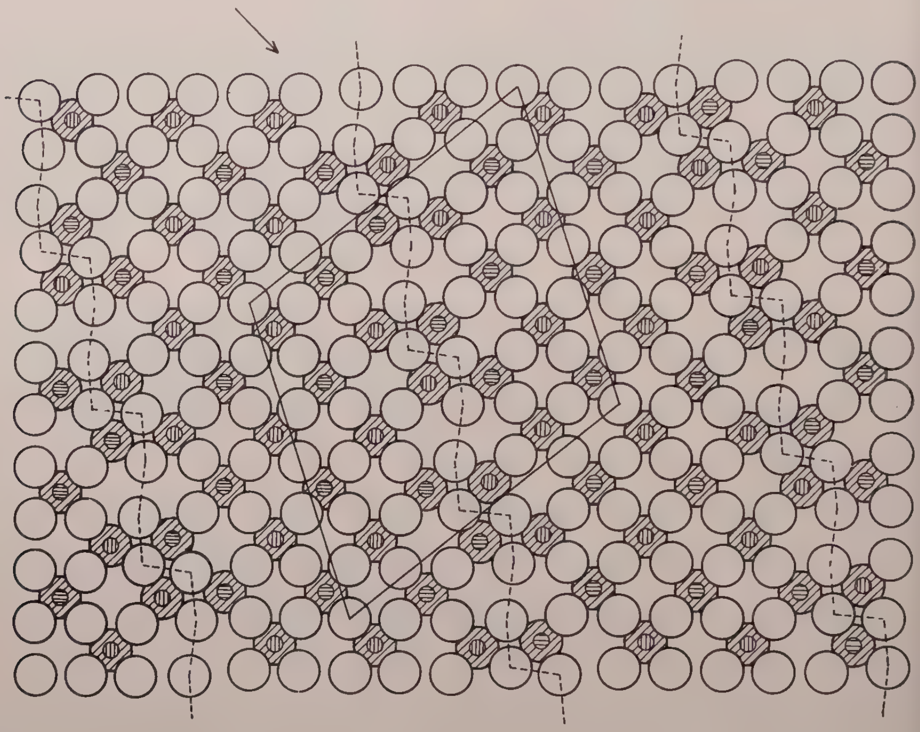
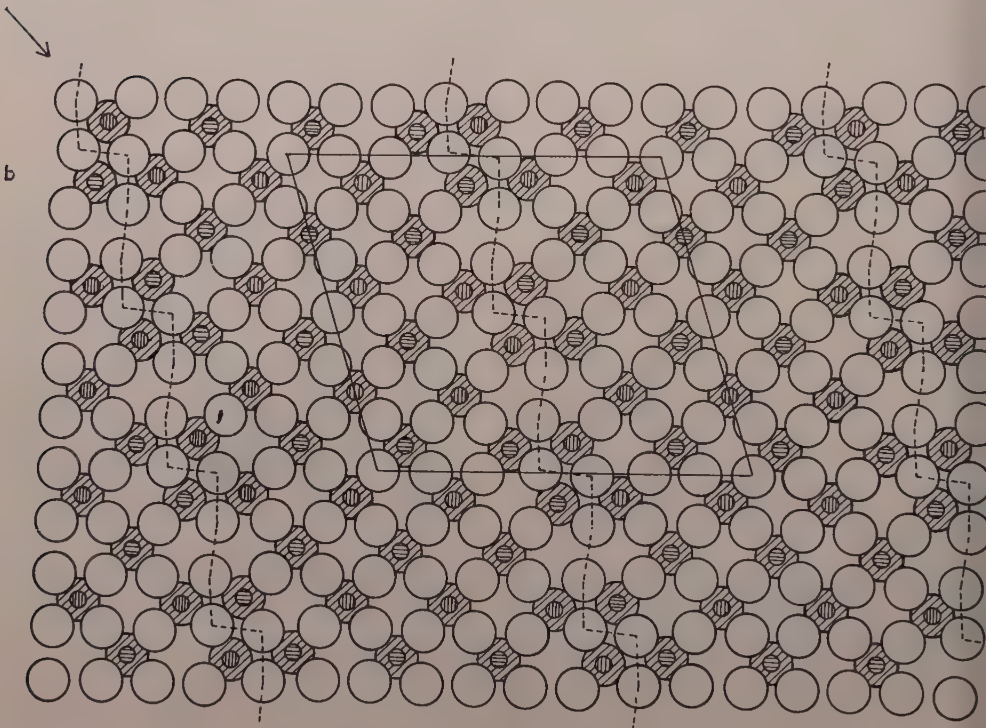
[1] The structure determination was carried out by L. K. and A. M.

a



b



Discussion

The aim of the present investigation was essentially to establish the formulae and structural types of the investigated substances while an accurate determination of the atomic positions was considered to be of minor interest as the proportion molybdenum : tungsten in the actual single crystals was not known with any certainty. No special precautions, *e.g.* use of multiple film technique, were thus taken for the determination of the intensities of the reflections. The relatively low number of terms in the Fourier series (especially for $(Mo,W)_{11}O_{32}$) is also likely to have affected the accuracy of the atomic positions derived. From this point of view the agreement between the observed metal atom positions and those derived for the ideal, hypothetic members $Me_{10}O_{29}$ and $Me_{11}O_{32}$ of the homologous series Me_nO_{3n-1} is surprisingly good, the differences for the x and z co-ordinates generally amounting to a few per mille of the axial lengths and never exceeding 0.007. (A detailed comparison between the observed and ideal structures of all the four known members of this series is given in (3).) It is also noteworthy that these deviations are of about the same magnitude as those found for Mo_8O_{23} and Mo_9O_{26}, where the metal atom positions were determined with a rather high degree of accuracy on the basis of the considerable number of reflections registered by means of Mo-K radiation.

The interatomic distances observed in $(Mo,W)_{10}O_{29}$ and $(Mo,W)_{11}O_{32}$ are, within the limits of probable error, compatible with the values previously obtained for several molybdenum and tungsten oxides and tungsten bronzes (7). Thus in $(Mo,W)_{10}O_{29}$ the metal–metal distances within the networks parallel to (010) are 3.71–3.88 Å for polyhedra joined by corners and 3.12 and 3.26 Å for polyhedra having edges in common, while the corresponding values for $(Mo,W)_{11}O_{32}$ are 3.63–3.87 Å and 3.26 Å. The distances parallel to the monoclinic axis are 4.00 Å in both double oxides.

Further investigations have been started in order to identify higher members of the series Me_nO_{3n-1} and to establish the influence of the value of n and of the proportion Mo : W on the structural details of the various homologues.

SUMMARY

The formulae and crystal structures of two molybdenum tungsten oxides have been determined by means of X-ray crystallographic methods. The structural properties of the two compounds, $(Mo,W)_{10}O_{29}$ and $(Mo,W)_{11}O_{32}$, are in agreement with those predicted for the members $Me_{10}O_{29}$ and $Me_{11}O_{32}$ of the "homologous series" Me_nO_{3n-1} (3).

Fig. 1. The crystal structures of a) $(Mo,W)_{10}O_{29}$ and b) $(Mo,W)_{11}O_{32}$. The structures are built up of blocks of distorted ReO_3-type, which may be described as consisting of MeO_6 octahedra joined by corners. The blocks are cemented to each other by octahedra sharing edges along folded contour planes, the projections of which are indicated by broken lines. The characteristic finite extensions of the blocks of ten and eleven MeO_6 octahedra are denoted by arrows. Vertically and horizontally shadowed metal atoms are mutually displaced in the direction normally to the extension of the figure, the metal atoms thus forming a puckered network. The oxygen atoms nearest to the observer are omitted.

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Institute of Chemisty, University of Uppsala, January 1953.

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ARKIV FÖR KEMI Band 6 nr 12

Vorgelegt am 14. Januar 1953

Versuche über Chinonmethide und o-Chinone

Von Hans von Euler und Hans Hasselquist

Die in diesem Institut früher ausgeführten Versuche an Chinonmethiden bezogen sich auf ihre Rolle bei der Resolhärtung, also bei dem in der Wärme stattfindenden Übergang von Resiten in Resole.[1] Dass bei diesem Komplex von Vorgängen die Chinonmethide als Zwischenprodukte auftreten steht auch mit den Ergebnissen von K. Hultzsch[2] in vollkommener Übereinstimmung. Obwohl die Chinonmethide seit den Arbeiten von K. v. Auwers und von Th. Zincke[3] und ihren Schulen Gegenstand eingehender Studien gewesen sind — es seien nach den Untersuchungen von R. Pummerer und Cherbuliez[4] besonders die von A. Zinke und von K. Hultzsch genannt, sind die an denselben verlaufenden Reaktionen nur unvollständig bekannt, und auch die Strukturen der polymeren Chinonmethide können kaum als endgültig festgestellt betrachtet werden. Vom Standpunkt der Elektronentheorie enthalten die Chinonmethide noch eine Reihe von Problemen, auf die wir an anderer Stelle zurückkommen.

Die oxydierende und reduzierende Wirkung von Chinonmethiden zeigt sich an mehreren chemischen Vorgängen, z. B. an der Dehydrierung von Phenolalkoholen zu Phenolaldehyden. Die o-Chinonmethide entsprechen in dieser Hinsicht, wie auch bezüglich der Tendenz zur Dimerisation in gewissem Grad den in zwei Formen auftretenden o-Chinonen.

Benzo-o-methylenchinon und Naphto-o-methylenchinon

1907 schrieben Fries und Kann:[5] Dass o-Methylenchinone existenzfähig sind ergibt sich aus den Untersuchungen von Fries aund Hübner[6] über das 1-Methyl-2-Naphtol. Dieses, wie auch sein Monobromderivat, werden durch salpetrige Säure leicht in das 1,2-Naphto-methylenchinon bezw. das 6-Brom-1,2-naphto-methylenchinon umgewandelt.

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[1] Euler, E. Adler und J. O. Cedwall, Arkiv f. Kemi etc. 14A Nr. 14; 1941 — E. Adler, Euler u. J. O. Cedwall, ebenda 15A Nr. 7; 1941 — Euler, E. Adler, G. Eklund u. C. Törngren, ebenda 15B Nr. 9; 1941. — Euler, E. Adler, J. O. Cedwall u. O. Törngren, ebenda 15A Nr. 11; 1941 — Euler, E. Adler u. A. O. Caspersson, ebenda 16, Nr. 11; 1943. — Euler, Angew. Chemie, 54, 458; 1941.

[2] K. Hultzsch, Ber. 74, 898, 1533, 1539; 1941. — 75, 106; 1942. — Monographie: Chemie der Phenolharze, Springer, 1950.

[3] Th. Zincke, u. C. v. Hohorst, Ann. 353, 357; 1907. — Siehe auch K. Fries u. K. Kann, Ann. 353, 335; 1907.

[4] R. Pummerer u. E. Cherbuliez, Ber. 52, 1392; 1919.

[5] K. Fries u. K. Kann, Lieb. Ann. 383, 339; 1907.

[6] K. Fries u. E. Hübner, Ber. 39, 435; 1906.

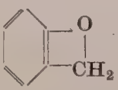
Beide Chinone sind aber im Gegensatz zu den von ZINKE und seinen Schülern in grosser Zahl beschriebenen p-Methylenchinonen auffallend indifferent: die für diese charakteristische grosse Additionsfähigkeit ist bei jenen nur schwach ausgeprägt.

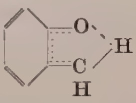
FRIES und KANN haben die von ZINKE und HEDENSTRÖM begonnenen Versuche zur Darstellung von o-Methylenchinonen der Benzolreihe fortgesetzt.[1]

PUMMERER und CHERBULIEZ[2] fanden Dehydro-methylnaphtol identisch mit dem Methylenchinon. Ihr Dehydro-methylnaphtol ist gegen Permanganat in indifferenten Lösungsmitteln (Aceton, Pyridin) empfindlich. Methylenchinon bezw. Naphtochinon-1,2-methid konnte von diesen Autoren in monomolekularer Form nicht gefasst werden, da es sich zu einer höher molekularen Verbindung polymerisiert. Diesen alkaliunlöslichen schwefelgelben Körper haben PUMMERER und CHERBULIEZ[3] krystallisiert erhalten; er ist, der Mol.Gewichts-Bestimmung zufolge durch Zusammenlagerung zweier Moleküle des Methylenchinons entstanden.

Die rasch verlaufende Dimerisierung des o-Chinonmethides ist verschieden formuliert worden, u. a. auch unter Hinweis darauf, dass nur eine Carbonylgruppe nachweisbar ist. So fassen PUMMERER und CHERBULIEZ (Ber. 52, 1396) o-Methylenchinon als einen cyklischen Dihydro-Körper auf.[4]

Chinonmethide werden ausser in der Chinonform auch in der Superoxydform geschrieben oder als Träger freier Radikale. In Betracht zu ziehen sind indessen auch Formulierungen, welche eine grössere Stabilität des Moleküls, z. B. gegen Hydrazine oder Hydroxylamin, anzeigen.





Wegen der grossen Tendenz der Chinonmethide zur Polymerisation ist es schwer, experimentelle Anhaltspunkte über Dissociations-Verhältnisse und Elektronen-Verteilung des Grundmoleküls zu gewinnen.

Der Verlauf der Reaktion der Methid-gruppe $-C=H_2$ mit Aldehyden (Benzaldehyd) ist noch nicht aufgeklärt.

Oxydation mit CrO_3 führt, nicht wie bei o-Chinonen, zu einer Ringspaltung; siehe S. 145.

I. Orientierende Versuche mit o-Chinonen

Um die Reaktionsfähigkeit der Chinonmethide mit derjenigen der o-Chinone zu vergleichen, wurden zunächst die folgenden Versuche mit letzteren angestellt, und zwar, um zu gut krystallisierenden Produkten zu gelangen, mit dem relativ stabilen *Tetrabrom-Chinon.*

---

[1] Über das p-Naphto-methylenchinon lagen keine diesbezgl. Angaben vor.

[2] R. PUMMERER u. E. CHERBULIEZ, Ber. 47. 2957; 1914.

[3] R. PUMMERER u. E. CHERBULIEZ, Ber. 52, 1932; 1919.

[4] Über die Bildung von o-Methylenchinon aus Xylenolalkohol und die Trimerisierung des Methylenchinons siehe E. ADLER, H. v. EULER u. J. O. CEDWALL, Arkiv f. Kemi etc. 15, Nr. 7; 1941.

Reaktion mit Dienen

Dass o-Chinone in analoger Weise wie p-Chinon (EULER u. JOSEPHSON[1]) mit Dienen reagieren, wurde von K. ALDER[2] beschrieben, dem man auch die ersten Synthesen von Dienen mit o-Chinonmethiden verdankt.

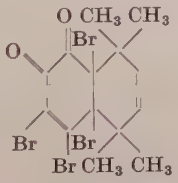
Eingehende Versuche über die Einwirkung von halogenisierten o-Naphtochinonen und o-Phenanthrenchinonen mit Dienen haben LOUIS F. FIESER und DUNN[3] ausgeführt.

A 1. Tetrabrom-benzochinon + Tetramethyl-butadien in der Wärme

1 g o-Chinon, in 2 ml Benzol gelöst, wurde mit 0,4 ml frisch dest. Butadiens versetzt. Bei der unter Wärmeentwicklung verlaufenden Reaktion nahm die dunkelrote Lösung schnell eine dunkel-blauviolette Farbe an (Chinhydronbildung des Reaktionsproduktes?), die im Verlauf von 10 Min. in Hellbraun überging. Nach Abkühlung mit Eiswasser krystallisierte das Produkt in Form farbloser Prismen aus, die abfiltriert und mit etwas Benzol gewaschen wurden. Das Rohprodukt (2 g vom Schmpt. 157–160°) aus Äthylacetat umkrystallisiert, ergab den Schmpt. 188–189° und lieferte nach weiterem Umkrystallisieren aus Benzol farblose Nadeln vom Schmpt 199–200°. Die Sbst. 173 ist sehr schwer löslich in Methonol, Äthanol und Wasser, leicht löslich in Benzol und Chloroform.

Analyse.

Ber. für $C_{14}H_{14}O_2Br_4$	C = 31,49 %	H = 2,75 %	Br = 59,87 %
Gefunden	31,68	2,35	59,53



A 2. Tetrabrom-benzochinon + Tetramethyl-butadien in der Kälte

1 g o-Chinon, in 2 ml Benzol gelöst wird unter Umrühren schnell mit 0,4 ml Butadien versetzt. Reaktion wie unter A 1 angegeben.

Das Produkt wird abgesaugt, vorsichtig mit Benzol gewaschen und dann aus kaltem Benzol umkrystallisiert, indem nur so viel Benzol angewandt wird dass der Hauptteil in Lösung geht. Der ungelöste grüne Rest wird abfiltriert. Nach Einengen des Filtrates scheidet sich ein weisser Niederschlag ab, der abfiltriert und vorsichtig mit Benzol gewaschen wird. Nach Wiederholung dieses Verfahrens ist die Substanz analysenrein. Schmpt 160–161°.

Die Sbst. ist schwerlöslich in Methanol, Äthanol und in Wasser, leicht löslich in Benzol, Äthylacetat, Chloroform und Aceton.

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[1] EULER und K. JOSEPHSON, Ber. 53, 822; 1920.

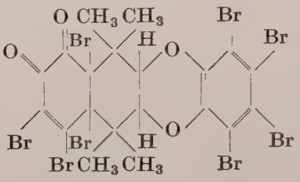
[2] K. ALDER und E. RÜDEN, Ber. 74, 920; 1941.

[3] LOUIS FIESER und J. T. DUNN, J. Am. Chem. Soc. 59, 1016, 1021, 1024; 1937.

Analyse.

Ber. für $C_{20}H_{14}O_4Br_8$ oder $C_{14}H_{14}O_2Br_4 + C_6O_2Br_4$	C = 25,05 %	H = 1,47 %	Br = 66.68 %
Gefunden	25,10	1,74	64,66

Es liegt also entweder eine Additionsverbindung der Sbst. 173 + Tetrabrom-benzochinon vor oder eine Verbindung der nachstehenden Struktur.



B. Tetrabrom-o-benzochinon + 1,4-Dimethyl-butadien[1]

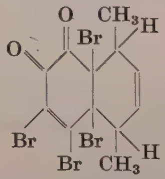
3 g Tetrabrom-o-Benzochinon in 6 ml Benzol gelöst wurden mit 1,1 g Butadien auf dem Wasserbad erwärmt. Während der Abkühlung schlug die rote Chinonfarbe der Lösung in gelb um. Die mit Methanol gefällte Sbst. 177 wurde abfiltriert und mit Methanol gewaschen. Das Rohprodukt (2 g, Schmpt 190–194°) wurde zweima aus Äthylacetat umkrystallisiert, wodurch 0,7 g mit einem Schmpt 198–199° erhalten wurde.

Sbst. 177 ist schwer löslich in Methanol, Äthanol und in Wasser, leicht löslich i Benzol, Äthylacetat, Chloroform und Aceton.

Der Mischschmelzpunkt mit Sbst. 173 (die selbst bei 199–200° schmilzt) betrug 170–173°, woraus sich die Verschiedenheit von Sbst. 173 und Sbst. 177° ergibt

Analyse.

Ber. für $C_{12}H_{10}O_2Br_4$	C = 28,49 %	H = 1,99 %	Br = 63,19 %
Gef.	29,24	2,35	62,09



Mit den aromatischen Aminen: Anilin, p-Nitranilin und β-Naphtylamin konnten wir au Terabrom-o-Benzochinon keine krystallisierten Produkte isolieren.

Oxydation der Sbst. 177 mit CrO_3. Sbst. 180

1,1 g Sbst. 177 wurden in 30 ml Essigsäure auf dem Wasserbad erwärmt und mit 2 g CrO_3 versetzt; die Oxydation trat dabei unter Gasentwicklung und Wärme entwicklung ein. Nach Beendigung der Reaktion wurde zu der abgekühlten Lösun Wasser in grossem Überschuss zugegeben. Das Reaktionsprodukt wurde dadurc als eine etwas schmierige Masse gefällt, die allmählich erstarrte und dann abfiltrier und mit Wasser gewaschen werden konnte. Nach zweimaligem Umkrystallisieren aus *wenig* Methanol wurde das Produkt 180 in Form kleiner, schwach violett-grauer viereckiger Blättchen vom Schmpt 163–168° erhalten.

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[1] Der entsprechende Versuch mit 1,3-Dimethyl-butadien, bei dem eine starke Wärmeent wicklung eintrat, lieferte das Reaktionsprodukt nicht in krystallinischer Form.

Analyse.

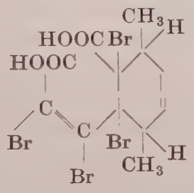
Ber. für $C_{12}H_{12}O_4Br_4$	C = 26,69 %	H = 2,24 %	Br = 59,21 %
Gefunden	25,31	2,17	59,50

Die Analyse liess schon vermuten, dass die Oxydation durch CrO_3 zu einer Aufspaltung des o-Chinonringes geführt hat, die wurde durch den folgenden Titrations-Versuch bestätigt.

20,5 mg Sbst. 180 wurden in 15 ml 96 %-igen Alkohols gelöst und mit 0,105-n NaOH bei 20° mit Phenolphtalein als Indikator titriert. Der Alkali-Verbrauch trat bis zum Zusatz von 0.77 ml NaOH schnell ein, hierauf äusserst langsam (0,03 ml in 15 Minuten), was vermutlich von einer langsamen Abspaltung (Verseifung) des Broms herrührte.

$$\text{Gef. E-Gew. bei 0,77 ml NaOH Verbrauch} = \frac{20,5}{0,77 \cdot 0,105} = 253$$

$$\text{Ber. für } C_{12}H_{12}O_4Br_4 \qquad 270$$



II. Versuche mit 2-Naphtochinon-1-methid

Darstellung des 2-Naphtochinon-l-methides

Diese Darstellung geschah im wesentlichen nach den Prinzip von K. **Fries** und E. **Hübner** über Di-2-naphtol-methan und 1-Methyl-2-naphtol. Wir verfuhren aber in folgender vereinfachter Weise:

Zur Gewinnung von *Di-2-naphtol-methan* wurden 100 g 2-Naphtol in 120 ml 96 %-igem Alkohol gelöst und mit 20 ml konz. Salzsäure + 55 ml 40 %igem Formaldehyd auf dem Wasserbad auf ca 60° erwärmt. Die Reaktion kam dadurch in Gang und verlief dann bei Zimmertemperatur innerhalb etwa 10 Minuten, worauf die Reaktionsmasse zu einem Krystallkuchen erstarrte, der mit Methanol-Wasser (1:1) verrührt und dann abfiltriert wurde. Ausbeute 92 g, Schmpt. 198–199°. Mischshmelzpunkt mit der nach **Fries** und **Hübner** dargestellten Sbst. ergab keine Depression.

Zur Darstellung von *1-Methyl-2-Naphtol* wurde das Di-2-naphtol-methan mit Zn-pulver alkalisch reduziert. Nach Fällung der Reaktionslösung mit HCl extrahierten wir die ausgefällte Masse nicht mit Wasser, sondern mit Benzol bei *Zimmertemperatur*, wobei das nicht in Reaktion getretene Naptholmethan ungelöst verbleib während das gebildete Methylnaphtol äusserst leicht in Lösung geht. Beim Einengen des Benzols auf ein kleines Volumen wurde das Methylnaphtol in Form langer farbloser Nadeln vom Schmpt 109–110° erhalten.

Das *Naphtochinonmethid* wurde nun aus dem Methylnaphtol mittels Natriumnitrit in essigsaurer Lösung dargestellt. **Fries** u. **Hübner** gaben eine Reaktionszeit von 9 Stunden an, während **Pummerer** und **Cherbuliez** nur 2 Stunden benötigten (Ber. 47, 2968; 1914). Das folgende Verfahren lieferte uns in kürzerer Zeit gute Ausbeuten:

1 g Methylnaphtol in 12 ml Eisessig gelöst wurde mit 10 ml Wasser versetzt, dem im Verlauf von etwa 5 Minuten 1 g Natriumnitrit zugegeben wurde. Dabei fällt das Methid in etwas schmieriger Form aus. Nach dem Nitritzusatz fängt man das Reaktionsprodukt mit einem Glasstab auf und wäscht es mit etwas Wasser. Nun wird die pappige Masse mit ca 1 ml Äthylacetat verrieben und geht dabei allmählich in Lösung, während das "Methid" in Form gelber, schwach grünlicher Nadeln vom Schmpt 133–134° auskrystallisiert. Ausbeute: 500 mg.

Das Naphtochinonmethid wurde mit den typischen Chinonreagenzien Phenylhydrazin, Semicarbazid, ferner mit Anilin und mit p-Phenylendiamin in Methanol bezw. in Benzol in Reaktion gesetzt. Es trat offenbar die schon von PUMMERER und CHERBULIEZ (Ber. 47, 2970) erwähnte Spaltung des polymeren Methides im Lösungsmittel ein. Dass die darauf folgenden Reaktionen zu keinen krystallisierenden Produkten führten, dürfte darauf beruhen, dass das Methid nicht als einheitliches Ausgangsmaterial vorlag.

Einwirkung von Diazomethan auf Naphtochinonmethid

300 mg umkrystallisiertes Naphtochinonmethid wurde mit etwas Methanol + überschüssigem, in Äther gelöstem Diazomethan versetzt, wobei das Methid allmählich unter schwacher Stickstoff-Entwicklung in Lösung ging. Nach 24 Stunden wurde bei Zimmertemperatur im Vacuum eingeengt bis ein intensiv gelb gefärbtes Öl zurückblieb, das in möglichst wenig Benzol gelöst und dann mit Petroleumäther gefällt wurde. Die überstehende klare, schwach gelbe Lösung wurde abdekantiert. Mit der Fällung wurde das gleiche Verfahren noch zweimal wiederholt, wobei ein intensiv gelbes, amorfes Produkt erhalten wurde, das abfiltriert und mit Petroleumäther gewaschen bei 100–106° schmolz. Ausbeute 50 mg. Sbst. 178.

Die Gefrierpunkts-Erniedrigung (18,1 mg Sbst. in 1,054 g Eissesig) betrug 0,200°. Daraus ergibt sich ein Mol. Gew. von 335. Dieses deutet auf die dimere Form des Methids hin.[1]

Analyse HH 178 (gelbes, amorphes Pulver).

Ber. für $C_{10}H_{10}O_2$	C = 74,1 %	H = 6,18 %
Gefunden	74,17	6,31

Einwirkung von p-Nitranilin auf Naphtochinon-methid Sbst. 179

Der Versuch wurde angestellt, um eine eventuelle Oxydation der Methidgruppe $\cdot C{=}H_2$ durch die Nitrogruppe kennen zu lernen.

400 mg Naphtochinon-methid in 4 ml Benzol gelöst, wurden mit 400 mg p-Nitranilin auf dem Wasserbad bis zum Sieden erhitzt das dabei allmählich in Lösung ging. Nach halbstündigem Sieden trat ein krystallinischer Niederschlag auf, der nach einer Stunde heiss abfiltriert wurde. Ausbeute 120 mg goldglänzender Nadeln vom Schmpt 224–225°.

Aus 95 %igem Äthanol umkrystalliesiert stieg der Schmpt auf 235°. Sbst. 179.

Das Filtrat wurde mehrere Stunden gekocht, ohne dass eine Fällung entstand. Nach Abkühlung trat eine krystallinische Abscheidung auf, die noch freies p-Nitranilin u. s. w. enthielt. Die krystallinische Abscheidung war leicht löslich in Äthanol.

Die Sbst. 179 ist schwerlöslich in Wasser, Benzol und Petroleumäther, ziemlich löslich in Äthylacetat, Methanol und in warmem Alkohol.

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[1] PUMMERER fand, dass bei der Oxydation von 1-Methyl-2-naphtol durch nur 1 Atom H wegoxydiert wird und dass das entstehende Naphtochinonmethid somit als dimer anzusehen ist.

Analyse. HH 179.

	C	H	N
Gefunden	C = 54,02 %	H = 4,47 %	N = 18,41 %
Als nächstliegende Buttoformel wurde berechnet $C_{28}H_{28}O_2N_8$	54,15 %	4,51 %	18,05 %

Oxydation von Tetrabromchinon-methid. Sbst. 181

1,1 g des (dimeren?) Methides wurden in 30 ml Eisessig auf dem Wasserbad erwärmt und mit 2 g CrO_3 versetzt, wobei Gasentwicklung und Wärmeentwicklung eintraten. Wurde nach Beendigung der Reaktion Wasser im Überschuss zugegeben, so fiel ein Reaktionsprodukt als gelbes Pulver aus. Nach zweimaligem Umkrystallisieren aus wenig Chloroform erhielt man die Sbst. 181 in Form langer goldgelber Nadeln vom Schmpt 234–235° (Schmpt. des Ausgangsmateriales 130°). Ausbeute 40 mg reine Sbst.

Aus der Mutterlauge konnte noch weiteres Oxydationsprodukt isoliert werden.

Die Sbst. 181 ist schwerlöslich in Wasser, ziemlich löslich in Chloroform, leicht löslich in Eisessig.

Analyse HH 181. 3,5,6-Tribrom-2-methyl-benzochinon 1,4.

	C	H	Br
Ber. für $C_7H_3O_2Br_3$	C = 23,4 %	H = 0,84 %	Br = 66,9 %
Gefunden	22,7	0,46	65,7

(Vgl. AUWERS u. v. ERGGELET.[1])

Bemerkungen zur Biochemie der Chinonmethide und Chinone

Über Auftreten und Wirkungen von Chinonmethiden im Tier- und Pflanzenkörper ist unseres Wissens nichts bekannt.

E. F. LEHMANN[2] entdeckte ausserordentlich starke zellteilungshemmende Wirkungen von Benzochinon, Naptochinon und Phenanthrenchinon, welche (an seinem Testobjekt) den Effekt des Colchicins noch übertrafen. Ob die antibiotische Wirkung welche o-Oxy-benzylalkohol in Bakterienkulturen ausübt mit dem intermediären Auftreten von o-Chinonmethiden in Beziehung gesetzt werden kann, ist unsicher.

Schon wegen der hohen Polymerisationstendenz der o-Chinonmethide und der sehr geringen Wasserlöslichkeit der Polymerisate werden diese o-Methide sehr selten eine antimitotische oder anticanceröse Rolle spielen.

Um so auffallender ist das Ergebnis unserer mit 2-Naphtochinon-1-methid angestellten Versuche, die allerdings erst in geringer Anzahl vorliegen.

6 weisse Ratten unseres norwegischen Stammes erhielten per Tier 10 mg 2-Naphtochinon-1-methid durch Injektion jeden zweiten Tag. 10 bezw. 20 Tage nach Impfung und Injektion wurden die folgenden Grössen der Sarkome gemessen:

	10 Tage	20 Tage
6 Versuchstiere (Mittel)	34 ccm	47 ccm
6 Kontroll-Tiere (Mittel)	18 ccm	22 ccm

Es zeigte sich also schon nach 10 Tagen eine starke Erhöhung des Sarkom-Wachstums.

Stockholm, Universität, Vitamin-Institut und Institut für organ.-chemische Forschung.

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[1] AUWERS u. v. ERGGELET, Ber. 32, 3033; 1899. — ZINCKE u. KLOSTERMANN, Ber. 40, 680 1907.

[2] E. F. LEHMANN, Experientia, 3, 233; 1947.

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Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

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Studies on the uptake of thorium B (lead) by the erythrocytes of the rabbit blood

By K. K. Reddi

With 1 figure in the text

Both in vivo and in vitro studies on the distribution of lead in the blood fractions were made by several workers. Schmitt and Taeger (1927), Teisinger (1936), Tompsett and Anderson (1941) found that the lead is equally distributed between red cells and plasma. Contrary to the above findings Blumberg and Scott (1935), Willoughby and Wilkins (1938) Bamach, Kehoe and Logan (1942) found upto about 90 % of the total lead in the erythrocytes.

This divergence in the results could be attributed to the lack of sensitivity in the analytical procedures employed by the above workers. Studies made by employing sensitive procedures, namely, using lead salts labelled with thorium B (Behrens and Pachur, 1927) and radium D (Mortensen and Kellog, 1944), showed that lead is mainly held by the erythrocytes. Recently the extensive studies made by Hevesy (1952), Alexander (1952), Hevesy and Nylin (1952) with the erythrocytes labelled with thorium B by leading an oxygen stream containing thoron through blood samples, showed that thorium B is held by the erythrocytes upto about 99 %.

The above studies definitely show that the lead is mainly fixed by the erythrocytes. But no attempt was so far made to isolate the component or components in the erythrocytes responsible for the fixation of the lead. The present communication deals with the studies relating to (i) the distribution of thorium B in various fractions of blood, (ii) stability of the thorium B fixed by the erythrocytes and (iii) isolation and characterization of the substances in the erythrocytes responsible for the uptake of thorium B.

Experimental

Materials and methods

Blood: Rabbit blood obtained by heart puncture was used in the experiments described below. It was collected into tubes containing heparin and was used immediately.

Activating blood with thorium B: The method of activating blood with thorium B was essentially the same as that described by Alexander (1952). An oxygen stream striking the surface of the radiothorium was led into freshly drawn rabbit blood.

Preparation of samples for measurement of radioactivity: The samples were dried thoroughly at 90° and powdered in an agate mortar.

Measurement of radioactivity: The dried material was spread to a thickness of 0.4 mm in micro aluminium discs, placed in brass cups. They were weighed and activities were measured in Geiger counter, having G.M. tubes with 2 mg/cm^2 mica windows.

Since the thoron and thorium A have short life times of 55 sec. and 0.145 sec. respectively, no radioactive substances other than thorium B and its disintegration products were present in the samples under the conditions of the experiments described below.

Distribution of thorium B in the plasma, stroma and hemolysate

25 ml blood was activated for 30 min. at room temperature.

(i) 10 ml portion was immediately withdrawn and centrifuged. The plasma was separated and dried. The erythrocytes were washed three times with an equal volume of 0.9 % NaCl and hemolysed with 6 times their volume of water and centrifuged. The stroma was washed with 0.9 % NaCl till the last washings were colourless and dried. An aliquot of the hemolysate was also dried at the same temperature. The activities of plasma, stroma and hemolysate were measured and the results are given in Table 1.

Table 1

Distribution of thorium B in plasma, stroma and hemolysate.

Material	Without shaking			After shaking		
	Counts/ mg/min.	Total activity	Per cent of total activity	Counts/ mg/min.	Total activity	Per cent of total activity
Plasma (from 1 ml blood)..	128	5619	25.4	38	1668	6.9
Stroma (,, 1 ,, ,,)..	316	632	2.9	287	574	2.4
Hemolysate (from 1 ml blood)	132	15840	71.7	183	21960	90.7

(ii) Another 10 ml portion was shaken gently for 30 min. at room temperature and centrifuged. The plasma was withdrawn and dried. The stroma and hemolysate were prepared in the same way as described above. The results are given in Table 1.

Fractionation of stroma

It is well known that stroma of the red cell is not a single entity, but is composed of mainly protein, phospholipid and cholesterol. It is of interest to know which of these components binds lead.

Procedure: 40 ml blood was activated for 30 min. and shaken gently for another 30 min. at room temperature. The plasma was separated by centrifugation. The red cells were washed thrice with cold 0.9 % NaCl and hemolysed by adding 6 times their volume of cold distilled water and centrifuged. The stroma was washed with cold 0.9 % NaCl till the last washings were colourless. It was then freeze dried. This preparation was fractionated in the following way.

Extraction with ether: 25 mg of the dried stroma was refluxed with 10 ml of ether for 2 hrs. The contents were cooled and filtered. A portion of the residue was dried for activity measurements and the rest was used for the following extraction.

Extraction with ethanol-ether mixture (3 : 1): 15 mg of the above residue was refluxed with 25 ml of ethanol-ether mixture on a water bath for 6 hrs. The contents were cooled and filtered. The residue was dried and its activity was measured.

The results are presented in Table 2 a.

Table 2 a

Distribution of thorium B in stroma.

Material	Counts/mg/min.
(i) Stroma	492
(ii) Stroma after extraction with ether	522
(iii) Residue from (ii) after extraction with ethanol-ether	566

Separation of cholinesterase from stroma: PALÉUS (1947) reported that the erythrocyte cholinesterase is attached to the stroma of the red cell. The procedure of AUGUSTINSSON (1948) was used by the author to separate the cholinesterase from the stroma.

40 ml blood was activated and stroma was prepared in the manner described above. 20 mg of the stroma was suspended in 8 ml distilled water and 2 ml 0.1 % lysolecithin in 1.26 % $NaHCO_3$ were added. It was shaken for 1 hr. and centrifuged. The residue and an aliquot of the supernatant were dried and their activities were measured. The results are given in Table 2 b.

Table 2 b

Distribution of thorium B in the stromatin and cholinesterase.

Material	Counts/mg/min.
Stroma	528
Cholinesterase	543
Stromatin	463

Stability of thorium B in the hemolysate

20 ml blood was activated for 30 min. and shaken for another 30 min. at room temperature. The hemolysate was prepared from the active washed red cells as described in the above experiments.

5 ml of the hemolysate was dialysed against 200 ml of the solutions listed in Table 5 for 24 hrs. at 0°. The outer contents were stirred occasionally. After dialysis, the volume was made upto 10 ml in each case with solutions against which it was dialysed. The results are presented in Table 3.

Table 3

Stability of thorium B in the hemolysate.

Solutions against which dialysed	Per cent retention of thorium B
Hemolysate (before dialysis)	100
,, after dialysis against distilled water	61
,, ,, ,, ,, 0.9 % NaCl	55
,, ,, ,, ,, borate buffer, pH 10.0.	79
,, ,, ,, ,, acetate buffer, pH 2.8	8

Separation of the active components in the hemolysate

The active blood was centrifuged and the plasma was removed. The erythrocytes were washed thrice with an equal volume of cold 0.9 % NaCl. The washed erythrocytes were chilled. To the chilled mass an equal volume of cold distilled water and an equal volume of ether were added and mixed well and centrifuged. The clear hemolysate was drawn out with a capillary.

(a) Ionophoresis.

Procedure: The ionophoresis was carried out according to the procedure of DURRUM (1950) in an apparatus of L. K. B. Type 327 A. Filter sheet (Munktell OB) of size 42 × 15 cm, soaked with phosphate buffer of pH 6.87 was allowed to dip with its ends into buffer reservoir fitted with electrodes. 0.01 ml drops of the active hemolysate were placed at the centre of the paper at 4 cm apart and covered with a glass sheet to prevent evaporation. The current, 2.5 ma. was passed for 15 hrs.

The spot spread to a diameter of 2 cm. The papers were dried at 100°. Two horizontal lines at a distance of 1.25 cm from the centre of the spot were drawn with a pencil and a strip of length 12 cm was cut. This was cut into strips of 1 cm width. Each small strip of size, 2.5 × 1 cm was further cut into three equal pieces and they were placed one over the other in an aluminium disc (depth, 2.5 mm and diameter, 13.7 mm). The activities were measured in the usual way.

The proteins were detected by using the staining method of DURRUM (1950) modified by KUNKEL and TISELIUS (1951).

The results are given in Fig. I.

(b) Precipitation methods.

Precipitation with 25 % alcohol: To the hemolysate at 0°, one third its volume of absolute alcohol at –10° was added and mixed well. It was kept in the ice chest for 30 min. and centrifuged. The precipitate was washed with cold 25 % alcohol till the last washings were colourless. The precipitate was freeze dried (Fraction I).

Denaturation of haemoglobin: To the supernatant from above half its volume of ice cold chloroform was added and mixed well for 4 min. It was kept in the ice chest for 30 min. and centrifuged. The residue was dried at 90° and the supernatant was freeze dried.

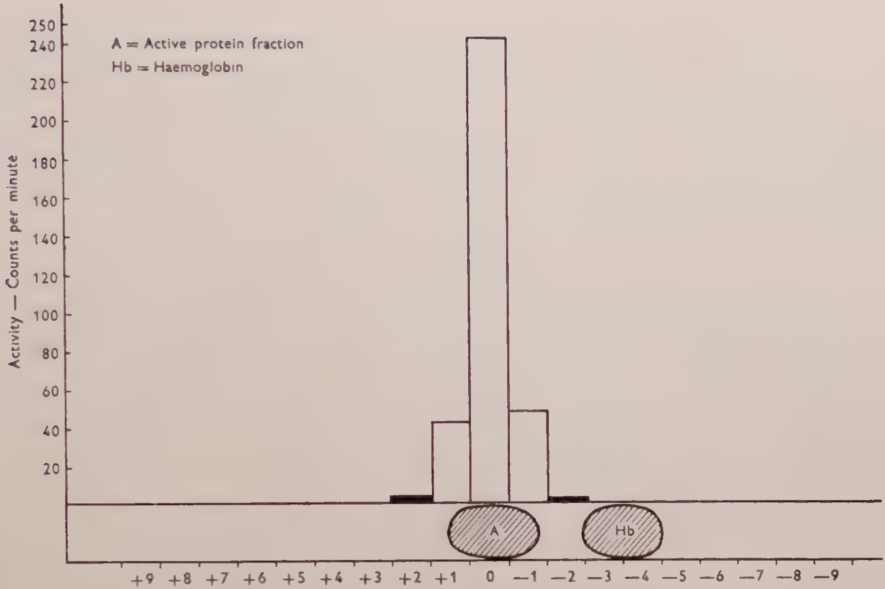

Fig. 1. Ionophoresis of the hemolysate in phosphate buffer pH 6.87.

Fractionation with 50 % acetone: The freeze dried material from above was taken in 4 ml cold distilled water and to this 4 ml of cold acetone were added. It was kept in the ice chest for 15 min. and centrifuged. The precipitate was dried at 90°.

An aliquot of the supernatant was also dried. The activities of the dried materials were measured and the results are presented in Table 4.

Table 4

Distribution of thorium B in the hemolysate.

Material	Counts/mg/min.	Per cent of total activity
Hemolysate	153	
Fraction I	2409	43.3
Residue (denatured haemoglobin)	58	18.6
Fraction II	392	2.1
Supernatant	13	0.2

Separation of the non-protein component from Fraction I

50 mg freeze dried material (fraction I) were dissolved in 10 ml N/50 NaOH and the protein was denatured by shaking with Sevag's reagent. It was shaken with 2 ml of the reagent, a mixture of chloroform and *n*-butanol (10 : 1), for 30 min. and centrifuged. This operation was repeated several times until the supernatant gave no reaction with ninhydrin.

The non-protein component was precipitated by passing CO_2 for 10 min. into the protein free supernatant solution. The precipitate, separated by centrifugation, was dissolved in 10 ml N/50 NaOH and a few drops of ammonia were added. To this 20 % lead acetate was added drop by drop till the precipitation was complete and centrifuged. The supernatant solution was treated similarly and centrifuged. The combined precipitates were washed 4 times with 5 ml distilled water each time and once with absolute alcohol. It was dried in vacuo over anhydrous $CaCl_2$.

The results are summarized in Tables 5 and 6.

Table 5

The distribution of thorium B in protein and non-protein components of Fraction I.

Material	Counts/mg/min.
Fraction I (in N/50 NaOH)	1036
Fraction I after extraction with Sevag's reagent (non-protein component)	726

Table 6

Precipitation of non-protein component with CO_2

Material	Counts/mg/min.
Non-protein component	601
Precipitate (obtained by passing CO_2 into solution containing non-protein component)	1113

Decomposition of lead salt with H_2S: 30 mg of the lead salt were suspended in 9 ml of distilled water. It was acidified with 6 N HCl and H_2S was passed into it till saturation and centrifuged. The supernatant solution was freed of H_2S by bubbling nitrogen into it.

Results

Distribution of thorium B in the plasma, stroma and hemolysate (Table 1): The plasma contained 25.4 % of the total activity immediately after activation and this was reduced to 6.9 % on shaking the activated blood. Stroma contained 2.9 % of the total activity and this was not much influenced by shaking.

Fractionation of stroma (Tables 2 a and 2 b): Thorium B was not bound by the phospholipid or the cholesterol of the stroma, but by its protein moiety. The results in Table 2 b show that thorium B was equally distributed between the stroma protein (stromatin) and the cholinesterase.

Stability of thorium B in the hemolysate (Table 3): When the active hemolysate was dialysed against borate buffer of pH 10.0, 21 % of the thorium B was lost, thereby showing that major part of thorium B in the hemolysate was bound by high molecular substances and the rest was either in a free state, fixed loosely or bound to low molecular substances. When the hemolysate was dialysed against water and 0.9 % NaCl, the loss in the activity was 39 % and 45 % respectively, showing that the splitting up of thorium B from its bound state takes place even at the neutral pH. The breakdown was almost complete when the hemolysate was dialysed against acetate buffer of pH 2.8

Separation of the active components by ionophoresis (Fig. 1): In phosphate buffer of pH 6.87, the haemoglobin migrated to negative side to a distance of 2.5 cm from the origin of the spot and this region showed no activity. The active material did not move and this region gave a positive protein reaction when treated with bromphenol blue reagent of DURRUM (1950) as modified by KUNKEL and TISELIUS (1951). When phosphate buffer of pH 8.05 was tried, the pattern remained the same. No buffer below pH 6.87 was tried, since the thorium B was found to be split off from its bound state in the acid medium.

Fractionation of hemolysate by precipitation methods (Table 4): Fraction I was found to be 15 times more active than the hemolysate of same weight. 43.3 % of the total activity of the hemolysate was present in the Fraction I. Thus the Fraction I formed 70 % of the non-dialysable thorium B present in the hemolysate. The material, obtained by freeze drying the Fraction I, was a light powder, light brown in colour. It was insoluble in water, mineral acids, 0.9 % NaCl but was soluble in N/50 NaOH upto about 50 %. The biuret, Millon-Nasse, xanthoproteic and ninhydrin tests were positive. It gave a green precipitate with Benedict's reagent, but no sugars were detected when the material was subjected to paper chromatography according to the method of PARTRIDGE (1948). It had a very weak blood group B specificity when tested according to the method of BRAY, HENRY and STACEY (1944).

The residue (the denatured haemoglobin) contained 18 % of total activity of the hemolysate. It was shown above that the haemoglobin did not take up the thorium B. The activity in the residue must be due to some non-haemoglobin component.

Fraction II contained 2.1 % of the total activity present in the haemolysate. It gave a positive protein reaction. Since the method of preparation of this fraction is similar to that described by TSUCHIHASHI (1923) for preparing crude carbonic anhydrase, the possibility of thorium B combining with this enzyme cannot be eliminated.

The supernatant from II showed a very negligible amount of activity.

Separation of non-protein component from fraction I (Table 5): On the basis of dry weight 1 mg of non-protein component was found to contain 2/3 of the activity present in 1 mg of fraction I. The results in Table 6 show that the active non-protein component could be precipitated completely by passing CO_2.

The analyses of the lead salt of the non-protein component, kindly performed by Dr. SIMON, Heidelberg, are given below:

C 35.95 H 4.88 N 12.14 Pb 26.08 per cent.

The supernatant solution, obtained by decomposing the lead salt with H_2S, was freed of H_2S by bubbling nitrogen into it and the following tests were carried out.

It gave a positive Millon-Nasse reaction and a white precipitate with bromine water. It gave an orange colour with diazotized *p*-nitroaniline (THEIS and BENEDICT, 1929) and a blue colour with phosphotungstic-phosphomolybdic reagent (RAKESTRAW, 1923). These tests go to show that the non-protein component of fraction I is most likely a phenolic compound.

SUMMARY

The distribution of thorium B in the plasma, stroma and hemolysate of rabbit blood activated with thoron was studied. It was found that thorium B was mainly held by the erythrocytes. The plasma contained 25.4 % of thorium B immediately after activation and this was reduced to 6.9 % on shaking the active blood. 2.9 % of thorium B was fixed by the stroma and this was not much influenced by shaking.

The stroma was fractioned to find which of its components was responsible for the fixation of thorium B. It was found that cholesterol and phospholipid portions of the stroma were free from thorium B. The thorium B was equally distributed between the stroma protein (stromatin) and the cholinesterase.

When the hemolysate was dialysed against borate buffer of pH 10.0, 21 % of the thorium B was lost. When it was dialysed against distilled water and 0.9 % NaCl the loss in the activity was 39 % and 45 % respectively. The loss of thorium B was almost complete when it was dialysed against acetate buffer of pH 2.8.

The precipitate, obtained by adding to the hemolysate 1/3 its volume of absolute alcohol, was found to be 15 times more active than the hemolysate of the same weight. It was responsible for the uptake of 70 % of the non-dialysable thorium B present in the hemolysate. The active material was composed of a protein and a non-protein component. The non-protein component is most likely a phenolic compound.

I wish to thank Professor G. de Hevesy for his valuable advice during the course of this investigation.

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Chelate alkali compounds in organic reactions. A general theory for the alkylation of metallic derivatives of tautomeric substances

By ARNE BRÄNDSTRÖM

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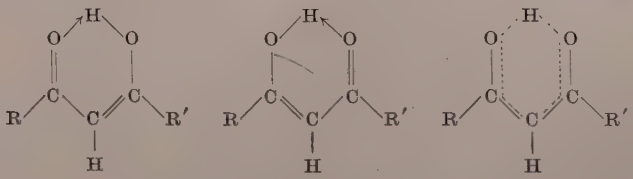
The alkylation of the sodium derivative of ethyl acetoacetate and related substances has been the subject of many investigations, and several theories have been put forward to explain why the sodium compound of ethyl acetoacetate, for instance, usually gives a C-alkylation compound, although on acidification at −78° it gives the pure enol-form of the ester (1). This problem is treated in practically every textbook in organic chemistry and a more thorough treatment has recently been published by H. Henecka (2). The situation is much more complicated than is usually supposed, however, and the present paper attempts to throw some light upon the factors operating and to arrange them in the form of a general theory.

The very extensive investigations hitherto published provide the following main facts:

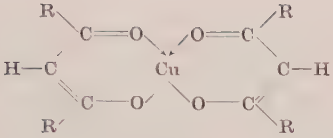
1. The alkali derivatives of tautomeric substances are often not typical salts but covalent compounds.
2. The reaction path depends on the nature of the solvent used, and in some cases is quite different in a polar solvent such as alcohol from that in a non-polar solvent such as ether or benzene.
3. The reaction path depends on the reactivity of the halide used.
4. The reaction path depends on the metallic derivative used. These factors will now be examinated more closely.

I. Salts or covalent compounds

1 a. The salt formulation. It is a generally accepted fact that the enol form of ethyl acetoacetate and related compounds will form a chelate ring (Formula I) with the aid of a hydrogen bond formation (3). A similar chelate formulation is also accepted for the multivalent metal derivatives of β-diketones, β-ketonic esters, o-hydroxy-aromatic esters and aldehydes, and o-nitrophenols. (Formula II). The alkali metal derivatives are, however, usually considered as salts. Henecka has ex-



Formula I

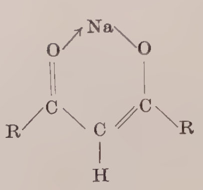


Formula II

pressed the alkylation reaction in the following way (4): "Nun ist doch der Natriumacetessigester als Salz einer wenn auch nur schwachen „Säure" mit einem Alkalimetall ohne Zweifel aus Ionen, dem Natrium-ion als Kation und dem verbliebenen sauren Rest als Anion aufgebaut. Betrachtet man den Reaktionspartner, beispiels das Halogenalkyl, so steht fest, dass ein solches Molekyl ausgesprochen heteropolar gebaut ist. In der Sprache der Elektronentheorie der Valenz bedeutet dies, dass das elektronenaffine Halogenatom die beiden die Bindung an den Alkylrest vermittelnden Elektronen weitgehend für sich beansprucht, wodurch das Molekül elektrisch unsymmetrisch polarisiert wird. Von da bis zur Ablösung des Halogenatoms als Ion im Augenblick der Reaktion mit dem ionogen spaltbaren Metallsalz einer β-Dicarbonylverbindung ist es daher nur ein kleiner Schritt. Dabei erscheint es gleichgültig, ob die teilweise Dissoziation des Natriumacetessigesters in Ionen im Reaktionsgemisch bereits vorgebildet war (alkoholische Lösung) oder ob die Dissoziation in reagierende Ionen erst im Augenblick der Reaktion mit dem Halogenalkyl (benzolische Suspension) eintritt. Ohne Zweifel wird bei der Reaktion das Metall als Kation abgespalten und zurück bleibt ein Anion, in dem ein innerer „Bindungswechsel", wie WISLICENUS sich ausdrückt, nun „um so leichter eintreten kann", als diese nur hinausläuft auf eine Verschiebung der Elektronen-Anteiligkeit der einzelnen Atome und Atomgruppen innerhalb des Anion im Augenblick der Reaktion mit dem nach Ablösung des Halogens als Ion verbliebene Kation des Reaktionspartners. Die Frage der reinen Strukturchemie nach dem Sitz des Natriums im Natriumacetessigester ist daher für die Frage der Deutung der Reaktionen dieses Salzes gegenstandslos geworden; geblieben ist die Frage nach der Electronenverteilung im Anion im Augenblick der Reaktion".

Apart from the contradiction between that the dissociation of the compound in a benzene solution does not occur until the reaction instant and the statement that the sodium derivative is a salt, the whole idea may be shown to be erroneous if it can be demonstrated that the alkali metal compound does not exist in an ionic form but in a covalent chelate form.

1 b. The covalent formulation. In a very interesting paper, SIDGWICK and BREWER (5) have described many alkali derivatives of tautomeric substances, and concluded that the generally accepted view that they are always salts must be greatly modified. They pointed out that if the alkali derivatives are salts they must have the usual properties of a salt of an organic acid: they will be insoluble in non-hydroxylic solvents and on heating they are likely to decompose (char) without melting. If they have the metal covalently attached to oxygen, we may expect non-polar properties; a definite melting point, and a distinct solubility in non-hydroxylic solvents. From the observations of the melting point and solubility in benzene, they classified a great many substances as salts and others as 2-covalent alkali compounds (Formula III).



Formula III

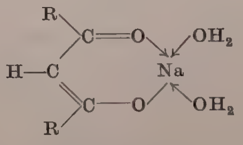
The *lithium* compound of ethyl acetoacetate, acetylacetone, benzoylacetone, o-nitrophenol, 3-nitro-o-cresol, and quinizarin are insoluble in toluene and decompose on heating without melting: they are thus salts. No 2-covalent lithium compound was found as a matter of fact.

Of the *sodium* compounds, those of ethyl malonate, acetylacetone, ethyl cyanoacetate, methyl salicylate, salicylaldehyde, o-nitrophenol, 3-nitro-o-cresol and quinizarin are salts. On the other hand, the sodium derivatives of ethyl acetoacetate, ethyl ethylacetoacetate, and ethyl ethanetetracarboxylate are 2-covalent compounds which are moderately soluble in non-hydroxylic solvents and have definite melting points. The sodium derivatives of alkyl and phenyl-malonic esters have been observed in 2-covalent modifications which were not stable but could be transformed into stable salts.

Of the comparatively few *potassium* derivatives examined, those of ethyl acetoacetate, ethyl cyanoacetate, and benzoylacetone are soluble in toluene and melt at 106°, 135–137°, and 146°, respectively, so that they are 2-covalent compounds.

The 2-covalent alkali compounds are by no means the only covalent alkali compounds observed. In fact a great many 4- and some 6-covalent alkali compounds were isolated. Those with the co-ordination number 4 are particularly stable, and many were prepared in the pure state. The following examples may be mentioned:

The sodium derivative of benzoylacetone ($C_{10}H_9O_2Na$) behaves as a salt: it has no definite melting point and is quite insoluble in hydrocarbons. The co-ordination of 2 moles of water gives the compound $C_{10}H_9O_2Na \cdot 2H_2O$. This compound melts at 115° and is somewhat soluble in benzene and chloroform, clearly indicating that it is not a salt but a covalent compound (Formula IV). A corresponding lithium compound was also obtained. Similar hydrates were obtained from the alkali derivatives of ethyl acetoacetate, acetylacetone, methyl salicylate, and salicylaldehyde. In fact, the formation of covalent addition compounds is such a usual reaction that most alkali derivatives which are insoluble in benzene and toluene readily dissolve in these solvents in the presence of excess of the mother substance. Thus it was found that sodium ethyl malonate, though quite insoluble in benzene, easily dissolved on warming in benzene containing an excess of ethyl malonate. Similar behaviour was observed with ethyl methylmalonate, ethyl cyanoacetate, acetylacetone, benzoyl-



Formula IV

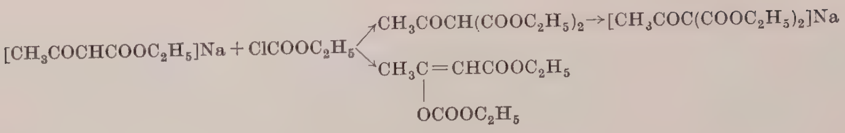
acetone, and ethyl acetoacetate. It is not always necessary that the parent substance should be present; for example, sodium acetylacetone will dissolve in a benzene solution of ethyl acetoacetate, and many compounds were isolated in which the alkali derivative of one compound had added another compound to form a well defined covalent chelate compound. It may be concluded from this investigation of SIDGWICK and BREWER that covalent alkali compounds are by no means exceptional, but on the contrary, quite common; their formation being strongly favoured by the addition of a polar compound such as water or a tautomeric compound. The formation of covalent compounds on the addition of other polar compounds such as alcohol or alkyl halides would of course be very difficult to establish, as the stability of such compounds would probably be too low for isolation in the pure state. But it is very likely that they do occur, especially when the reaction is carried out in a rather concentrated alcoholic solution. This possibility must therefore be taken into consideration in the alkylation reaction, and as this has not been done previously it is necessary to review the wide range of experimental facts in a wider content manner than customary hitherto.

II. O- and C-acylation

2 a. The effect of the solvent on the reaction path has usually not been considered in the previous theoretical interpretations, but this is not surprising, as the effect of the solvent is very hard to explain in terms of an ionic mechanism and as no reasonable alternative explanation has been put forward. However, if it is remembered that the addition of a polar compound will favour the formation of 4-covalent alkali derivatives, but in the absence of such a polar compound the alkali derivatives are salts or 2-covalent compounds, it might be expected that a study of the reaction in a polar and in a non-polar medium would give valuable information about the reaction mechanism.

HENECKA's statement that it is unimportant whether the reaction occurs in an alcoholic solution or in a benzene suspension, disagrees in many cases with the experimental facts. The most interesting example of the importance of the solvent is found in the reaction of the alkali derivatives of β-ketoesters with acid halides. It is well known that in this reaction both O- and C-acylation products may be formed, and it is of interest to see what conditions favour the domination of the one or the other reaction.

2 b. The reaction of sodium ethyl acetoacetate with ethyl chloroformate has been particularly well investigated and shows many interesting features. A. MICHAEL (6) says that he has studied the reaction in both ether and alcohol and has always obtained the O-carbethoxy compound. However, this conclusion is far from acceptable. NEF (7) reported that the usual method of adding water to the reaction product and examining the ether extract will cause the C-acylation product (diethyl acetylmalonate) to be overlooked as the mixture will be alkaline even after considerable reaction time (Formula V). This was explained by the fact that the C-acylation product is a rather strong acid and forms an alkali derivative, rather resistant to acylation, which passes into solution in the water layer when water is added. He did actually obtain the diethyl acetylmalonate on acidification. However, he held the erroneous view that the neutral product obtained from the ether phase was

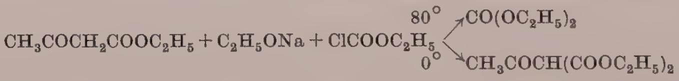


$$[CH_3COCHCOOC_2H_5]Na + ClCOOC_2H_5 \begin{array}{l} \nearrow CH_3COCH(COOC_2H_5)_2 \rightarrow [CH_3COC(COOC_2H_5)_2]Na \\ \searrow \begin{array}{l} CH_3C{=}CHCOOC_2H_5 \\ \quad | \\ \quad OCOOC_2H_5 \end{array} \end{array}$$

Formula V

not an isomeric compound but an acylation product of diethyl acetylmalonate. The incorrectness of this view was clearly demonstrated by Claisen and Zedel (8) who made an accurate analysis. Michael (9) also later came to the correct conclusion that both C- and O-acylation take place in an ether suspension, but that the O-acylation predominates.

Michael's statement (6) that he had obtained O-acylation in an alcoholic solution cannot be correct, however. Regarding the reactions in this medium, the following investigations may be mentioned. J. Wislicenus (10) stated that he did not obtain diethyl acetylmalonate from the reaction of ethyl acetoacetate with ethyl chloroformate in an alcoholic solution of sodium ethoxide, but that the product obtained was diethyl carbonate resulting from the reaction of ethyl chloroformate with the solvent. A. Pinner (11) had also obtained the same result on heating an alcoholic solution of sodium ethyl acetoacetate with ethyl chloroformate. But, on the other hand, Conrad and Geutzeit (12) stated that they had obtained the diethyl acetylmalonate in good yields from an alcoholic solution of sodium ethyl acetoacetate and ethyl chloroformate. These different results were clearly explained by M. Conrad and C. Brückner (13) as due to the fact that without heating the product will be diethyl acetylmalonate, and with heating diethyl carbonate (Formula VI). This



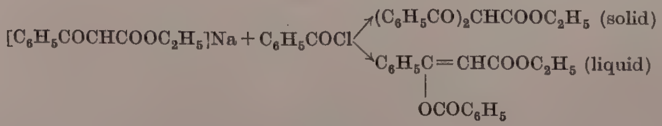
$$CH_3COCH_2COOC_2H_5 + C_2H_5ONa + ClCOOC_2H_5 \begin{array}{l} \overset{80^\circ}{\nearrow} CO(OC_2H_5)_2 \\ \underset{0^\circ}{\searrow} CH_3COCH(COOC_2H_5)_2 \end{array}$$

Formula VI

depends on the very different temperature coefficients of these two reactions. L. Claisen and W. Zedel (14) have checked this reaction, cooling the reaction flask in ice-water, and state that diethyl acetylmalonate was obtained in an almost quantitative yield.

From the facts mentioned above it can be concluded that the reaction will give mainly the O-acyl derivative in an ether suspension, but in an alcoholic solution the C-acylation product is the only one obtainable. That this is a quite ordinary effect can be demonstrated by another well investigated reaction.

2 c. The benzoylation of ethyl benzoylacetate. In an extensive investigation of the reactions of ethyl benzoylacetate, A. Bernhard (15) describes the reaction of sodium ethyl benzoylacetate with benzoyl chloride in an ether suspension. The reaction gave rather good yields of both C- and O-acylation products, in a proportion of about 2 to 1 (Formula VII). The same reaction was also studied by W. H. Perkin



$$[C_6H_5COCHCOOC_2H_5]Na + C_6H_5COCl \begin{array}{l} \nearrow (C_6H_5CO)_2CHCOOC_2H_5 \text{ (solid)} \\ \searrow \begin{array}{l} C_6H_5C{=}CHCOOC_2H_5 \text{ (liquid)} \\ \quad | \\ \quad OCOC_6H_5 \end{array} \end{array}$$

Formula VII

JUN. and J. STENHOUSE (16), but their results are usually interpreted as if they had not obtained any O-acylation product. This is a misunderstanding, however, as an inspection of their purification method clearly indicates that they lost the liquid O-acylation product on drying the reaction product on a porous plate, after which only the solid C-acylation product remained.

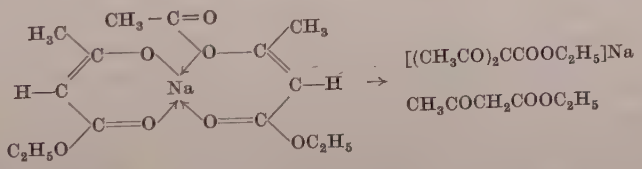
The same reaction has also been studied in an alcoholic solution by C. BÜLOW and E. HAILER (17). It is very interesting to note that their isolation process was such that they would have retained any O-acylation product which might be formed in an ether solution containing the C-acylation product. On evaporating the ether, however, they obtained the solid C-acylation product ethyl dibenzoylacetate in such a state of purity that it needed no further purification (Formula VIII). This clearly indicates that no O-acylation product was formed in the alcoholic solution although a considerable quantity was formed in ether suspension.

$$C_6H_5COCH_2COOC_2H_5 + C_2H_5ONa + C_6H_5COCl \rightarrow (C_6H_5CO)_2CHCOOC_2H_5$$

Formula VIII

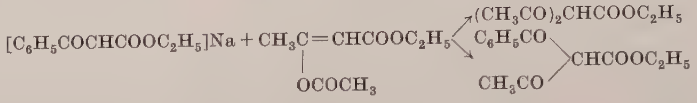
2 d. O- to C-rearrangement. In this connection another possible reaction must also be considered. It is well known that certain O-acylation products rearrange to become the C-acylation products, and this might perhaps be possible under the conditions used to prepare the acyl compound. This possibility has been particularly well investigated in the case of the isomeric acetyl derivatives of ethyl acetoacetate.

L. CLAISEN and E. HAASE (18) studied the reaction of ethyl O-acetylacetoacetate with certain basic catalysts, and stated that an isomerisation to the C-acetyl derivative is easily effected. One such basic catalyst is the sodium compound of ethyl acetoacetate, and another potassium carbonate, in which case a small amount of ethyl acetoacetate is stated to assist the catalysation. The rearrangement was considered as a C-acylation of the alkali derivative, the O-acetyl compound functioning as the acylation agent. The authors also made the very interesting observation that on heating equimolar amounts of sodium ethyl acetoacetate and ethyl O-acetylacetoacetate on a water bath the mixture first became fluid, after which the sodium derivative of the C-acetyl compound was deposited. Bearing in mind the work of SIDGWICK and BREWER (5) this can be explained as a formation of a low M. P. covalent derivative of the two initial reagents. The formation of the C-acetyl compound might then be the result of a reaction within the complex (Formula IX).



Formula IX

The fact that the rearrangement of the O- to the C-acyl compound is an acylation of an alkali compound of the β-ketoester with the O-acyl derivative was clearly shown by W. DICKMANN and R. STEIN (19). Their experiments showed that the C-acylation compound formed will not be more than equivalent to the amount of alkali metal added, and that the reaction of ethyl O-acetylacetoacetate with the sodium compound of ethyl benzoylacetate will yield a considerable quantity of ethyl acetylbenzoylacetate (Formula X).



$$[C_6H_5COCHCOOC_2H_5]Na + \underset{\displaystyle OCOCH_3}{\underset{|}{CH_3C}}{=}CHCOOC_2H_5 \begin{array}{l} \nearrow (CH_3CO)_2CHCOOC_2H_5 \\ \searrow \begin{array}{l} C_6H_5CO \\ CH_3CO \end{array} > CHCOOC_2H_5 \end{array}$$

Formula X

However, it is not correct to consider the formation of the C-acylderivatives as only due to a secondary product from the O-acyl derivatives. This was clearly demonstrated by A. MICHAEL (20), who showed that no reaction can be detected between equimolar amounts of sodium ethyl acetoacetate and ethyl O-acetylacetoacetate after standing in an ether solution at 0° for 1 hour. Under comparable conditions the reaction of acetyl chloride with sodium ethyl acetoacetate yielded the C-acetyl derivative as the main product. This fact indicates that the C-acylation product in this case had not been formed by a rearrangement of the O-acyl compound. An experiment with ethyl O-carbethoxyacetoacetate and sodium ethyl acetoacetate showed that no diethyl acetylmalonate was obtained after heating in an ether solution at 100° for 10 hours.

From what is said above, it is quite obvious that the O- and C-acylations are different processes, of which the C-acylation is the only one that will proceed in an alcoholic solution. The O-acylation, on the other hand, will be favoured by a nonpolar solvent.

We will now proceed to examine the various theories of the alkylation and acylation processes in the light of these experimental facts.

III. Alkylation theories

3 a. Eistert's theory. The difference between the alkylation of ethyl acetoacetate which always gives the C-alkylation compound and the acylation which gives considerable quantities of O-acylation products has been interpreted by B. EISTERT (21) in the following way: "Die ionische Auffassung der Salze im Verein mit der Mesomerie-Vorstellung lässt voraussehen, dass das El.-System im Anion, auch wenn es weitgehend der Enolstruktur entspricht, sehr leicht verschiebbar ist. Das Alkylierungsmittel, z.B. Jodmetyl, polarisiert sich im Grenzfall zu den Ionen J^- und CH_3^+, und die Annäherung des letzteren polarisiert die $C=C$-Doppelbindung des Enolats bzw. verschiebt die Mesomerie des gesamten Anions in Richtung auf die Ketonstruktur soweit, dass das CH_3^+ an das dann am C verfügbar werdende einsame El.-Paar treten kann.

Anderseits erhält man aus Na-Acetessigester und Chlorkohlensäure-ester ausschliesslich das Derivat der Enolat-Grenzform. Man darf den Grund für diesen andersartigen Reaktionsverlauf wohl in folgendem sehen: Das Oktett des Zentral-C-Atoms im Chlorkohlensäure-ester ist „desintegriert", weil es ausschliesslich von Schlüsselatomen beansprucht ist. Wenn nun bei der Reaktion das Chlor als Cl^- abionisiert, muss sich die am C verbleibene Lücke zwar wieder auffüllen, aber sie wird es möglichst in der Weise tun, dass wieder ein „instabiles" Oktett entsteht. Hierfür besteht nur die Möglichkeit, dass das Kation $[COOR]^+$ an den Sauerstoff tritt, wie er in der Grenzform zur Verfügen steht."

3 b. S_1 or S_2 mechanism? However interesting this explanation may be, it should be remembered that the reaction must be a replacement of the type $A^- + RX \rightarrow AR + X^-$ if it is assumed that the mesomeric anion enters into the reaction (the alkali compound as a salt). This type of reaction has been subjected to quantum theory considerations by HEITLER and LONDON (22), and the mechanism has been carefully investigated (23). The different types of possible reactions are classified under the S_1 and S_2 substitution mechanisms (24).

The theory put forward by EISTERT may be considered from this standpoint. In the explanation given by EISTERT, the reaction has been considered as taking place in two stages. The first one is a dissociation of the halide RX into R^+ and X^-. This stage will then be followed by the second stage $R^+ + A^- \rightarrow RA$. Reactions of this type are well known and extensively investigated and are of the S_1 type. The assumption of such a reaction type here leads to consequences at variance with the experimental findings, showing that the theory put forward by EISTERT must be erroneous. Firstly, the reaction must be of the first order, with the dissociation of the halide as the rate-determining step. This is contrary to the experimental facts as the reaction is definitely bimolecular (25). Secondly, the relative reaction rates of the halides would be *tert*-butyl > isopropyl > methyl. This is in direct contrast to the experimental facts.

3 c. Possible mechanisms. From what has been said above it must be concluded that the alkylation of the sodium compound of ethyl acetoacetate and related compounds is a bimolecular reaction proceeding in accordance with a S_2 substitution mechanism. The following two types of such mechanisms may be considered:

$$A^- + RX \rightarrow RA + X^-$$

$$MA + RX \rightarrow RA + M^+ + X^-$$

However, it will be shown that these formulations are very similar when certain postulates are made.

3 d. Dewar's theory. Let us first consider the reaction of the mesomeric anion. For the prediction of reaction rates, two alternative theories are available, *viz.* the collision theory and the transition state theory. The collision theory says that the reaction velocities at a selected temperature will be proportional to the collision frequency Z, of the two reagents, a steric factor P, and a factor $e^{-E/RT}$ where E is the energy of activation, R the gas constant, and T the absolute temperature. This has been used DEWAR (26) to explain the alkylation. We will consider his theory in some detail. He says: "The rate of addition will vary with the collision frequency and the entropy and energy of activation. Now a cationoid reagent will collide most frequently with that atom in a mesomeric anion which carries the largest net negative charge; and the entropy of activation will be least for the reaction which leads to least displacement of nuclei in the anion. Thus the temperature-independent factor A_b for formation of an enol derivative from an enolate ion is larger than the corresponding factor A_a for ketone formation; because in the ion the charge resides mainly on oxygen and the structure already approximates to $C = C - O^-$.

Therefore the velocity constants for ketone and enol formation are given by

$$K_{\text{ketone}} = A_a e^{-E_a/RT}$$

$$K_{\text{enol}} = A_b e^{-E_b/RT}$$

where $A_a < A_b$, $E_a < E_b$. The ratio of rates is therefore given by

$$\frac{K_{\text{ketone}}}{K_{\text{enol}}} = \frac{A_a}{A_b} e^{(E_b - E_a)/RT}$$

Now the difference in activation energies $(E_b - E_a)$ will presumably be roughly proportional to their absolute magnitudes. Therefore for very active cationoid reagents, i.e. compounds combining with the anion with low activation energies, $(E_b - E_a)$ will be small; the ratio of rates is then determined largely by the ratio A_a/A_b, and so enol is preferentially formed. But for a cationoid reagent of low activity, the difference $(E_b - E_a)$ will be large; the exponential factor will then outweigh the difference in the A's, and so ketone is formed more rapidly."

3 e. Criticism of Dewar's theory. From what he says above it must be concluded that DEWAR considers the fraction A_a/A_b as constant, independent of the cationoid reagent added. The factors A_a and A_b can, according to the transition state theory, be expressed as

$$A_a = X_a \frac{kT}{h} \cdot e^{S_a/R} \text{ and } A_b = X_b \frac{kT}{h} \cdot e^{S_b/R},$$

where X_a and X_b are the statistical mole fraction of the anion with the negative charge on the central carbon atom or on the oxygen atom resp., S_a and S_b are the entropies of activation, k the Bolzmann constant and h the Planck constant. The fraction will therefore be

$$A_a/A_b = X_a/X_b \, e^{(S_a - S_b)/R}$$

The fraction X_a/X_b will depend on the structure of the anion, and is usually considered as a function of the inductive and electromeric effect of the groups in the anion. This offers an explanation of why, for instance ethyl acetoacetate gives a greater proportion of O-acylation products than diethyl malonate under identical conditions. DEWAR supposes that the ionic charge of the anion resides mainly on the oxygen atom, that is $X_a/X_b \ll 1$. We will consider whether this agrees with the experimental facts. From what is said above, it can be seen that

$$\frac{K_{\text{ketone}}}{K_{\text{enol}}} = \frac{X_a}{X_b} \cdot e^{(S_a - S_b)/R} \cdot e^{(E_b - E_a)/RT}$$

From this equation it can be calculated that a change of 4.6 E.U. in $(S_a - S_b)$ or 1360 cal in $(E_b - E_a)$ at 25° will change the ratio of the C- to the O-substitution by a power of 10.

Unfortunately, the rate of both O- and C-alkylations or acylations have not been measured for the same compound, but rather good indications can be obtained from other measurements.

Table I

The energy of activation in the reaction of some sodium compounds with alkyl iodides.

Na-compound	Alkyl iodide	E
Ethylate	Methyl	19.49
Phenolate	»	20.60
o-Cresolate	»	20.35
m-Cresolate	»	20.54
p-Cresolate	»	21.25
Ethyl acetoacetate	»	18.94
Ethylate	Ethyl	20.65
Ethyl acetoacetate	»	18.71
Ethyl ethylacetoacetate	»	18.60
Ethyl allylacetoacetate	»	19.07
Ethyl acetoacetate	Propyl	18.91

In an alcoholic solution, only C-alkylation and acylation products have been obtained with sodium ethyl acetoacetate. As the reaction has been extensivly investigated this must correspond to a value of the fraction of $K_{\text{ketone}}/K_{\text{enol}}$ which is > 100, since 1 % of the O-product would certainly have been detected in the saponification of the C-product. This corresponds to a value for $(E_a - E_b) \gg 2720$. The activation energy measured for the methylation of sodium ethyl acetoacetate with methyl iodide is 18.9 Cals. The corresponding value for the O-methylation reaction must then be $\gg 21.6$ Cals. This value is, however, somewhat too high in comparison with the values obtained for the O-methylation of phenols and alcohols (Tab. I) (27), which are in the region 20.1–21.3 Cals. It seems then very likely that a value for the difference $(E_a - E_b) \gg 2700$ cal, which must hold even for the acylation reaction will be somewhat higher than the value to be expected in view of the experimental results available. The different proportions of the C- and O-acylation products of ethyl acetoacetate when the acylation is carried out in alcoholic solution and in ether suspension provides further evidence against DEWAR's theory. The effect of the solvent on the rate of a reaction has been explained by many authors (28) and arranged by DEWAR (29) in the form of a general theory. According to this theory the solvent stabilizes ions because molecules of solvent are electrostatically attracted to them, and the degree of stabilization will depend on the total electrostatic energy of the shell of solvent molecules surrounding the ion. Now the attachment of the solvent molecules to the ions will be accompanied by a distortion of their electronic orbitals and a large decrease in energy. This energy decrease will lower the activation energy if this solvation energy is higher for the transition state than for the initial state, and vice versa. For the reaction

$$A^- + RX \rightarrow A^{\delta -} \cdots R \cdots X^{\delta -}$$

the total change is the same in both states, but in the transition state it is shared between A and X. Now the energy of solvation of a charge does not depend only on its magnitude; the total solvation energy of two separate small charges is less than the solvation energy of a charge twice as great. Therefore, the solvation energy of the transition state is less than that of the initial state, and since the solvation energy increases with increasing dielectric constant of the solvent, the result will be that the activation energy is higher for a reaction of this type in a polar solvent than in a nonpolar one.

According to DEWAR the difference in activation energies ($E_b - E_a$) will be roughly proportional to their absolute magnitudes. Hence this difference must be larger in a polar solvent than in a nonpolar one, but this means that a greater proportion of C-acylation products should be obtained in alcoholic solution than in benzene or ether suspension (or solution, since many alkali compounds of β-ketoesters are soluble in benzene). This is in qualitative agreement with the experimental facts, but the effect is much greater than expected. It must be remembered that if the acylation reaction is of the type $A^- + RX \rightarrow AR + X^-$ for both the O- and the C-substitution, as has been assumed by DEWAR, the effect of the solvent must be the same for both reactions. The fact that in the reaction of ethyl chloroformate with sodium ethyl acetoacetate the product is mostly the O-acylation compound if the reaction is carried out in ether, but exclusively the C-acylation one if it is carried out in alcohol must correspond to a change in the ratio of O- to C-compound of more than 10^2, which in its turn corresponds to a difference in the activation energy of more than 2720 cal. arising from the difference in solvation energy in both reactions. This value seems to bee much too high if the O- and C-acylation reactions are considered to be totally analoguous.

Besides these not very serious objections to the theory given by DEWAR, there are other facts that cannot be explained at all by his theory. Of these the following may be mentioned here:

No O-methylation compounds are obtained when the sodium compounds of ethyl acetoacetate and acetylacetone are used, but considerable quantities are obtained with the silver compounds (30).

Halogen compounds which are not very reactive may sometimes give O-alkylation products. The most interesting of such reagents are the bromoacetaldehyde diethylacetal (31) and the β-dialkylamino ethyl chlorides (32).

Lastly, some cyclic β-diketones and ketoesters give a high proportion of O-alkylation products with the simple alkyl halides, whereas the open-chain ones give exclusively C-alkylation products.

These and some other effects will be more closely examined later and a plausible explanation given.

3 f. Conclusions. From the calculations made above it must be concluded that the variation in ($E_b - E_a$) cannot explain all the facts. There must therefore be a variation in

$$A_a/A_b = X_a/X_b\, e^{(S_a - S_b)/R}$$

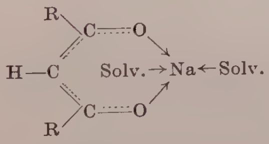
The fact that more C-alkylation is obtained than expected can be explained by the factor ($S_a - S_b$) only if $S_a > S_b$, but this means that a reaction with the carbon atom would be sterically more favoured than a reaction with the oxygen atom. This is in direct contrast to the effect which can be expected. The explanation must therefore be that the fraction X_a/X_b is not $\ll 1$ in an alcoholic solution but much higher. Furthermore, this fraction cannot be taken to be constant; it varies with the experimental conditions. The latter is in fact only what is to be expected, and was clearly recognised by EISTERT (21), although he applied it incorrectly.

IV. The alkylation mechanism for metallic compounds of β-ketoesters and related substances which are sterically capable of forming simple chelate compounds

4 a. The structure of the alkali derivatives in solution. The reaction in alcoholic solution will now be examined in the light of the facts known about a reaction of the type $A^- + RX \rightarrow AR + X^-$, and a theory will be deduced which, as far as the author can see, is capable of explaining all the experimental facts.

In all ionic mechanisms it must be remembered that all ions are solvated, and that a metal cation is never far away from a negative charge. The existence of sodium compounds of the type $NaBO_2 \cdot 4H_2O$, $Na_3PO_4 \cdot 12\ H_2O$, $Na_3AsO_4 \cdot 12H_2O$ etc. suggests the existence of the $[Na(H_2O)_4]^+$ cation (33). In alcoholic solution a similar ion containing C_2H_5OH instead of H_2O seems probable. It must further be remembered that the solvent stabilizes ions because molecules of solvent are electrostatically attracted to them, and in some cases stable co-ordination compounds may result. It is, however, not only solvent molecules that are attracted; all molecules in the solution are attracted to some extent. Sometimes this attraction of molecules other than the solvent may give rise to considerable affects. To give a concrete example, the ether formation from benzhydryl chloride in alcohol is greatly accelerated by small amounts of water (34). In a solution of sodium ethyl acetoacetate we have the two oxygen atoms in the anion, which may be attracted to the metal ion just as the solvent molecules are. In the case of multivalent metals this attraction will give rise to the formation of chelate compounds, and the existence of such compounds even with the alkali metals has been pointed ~~out~~ in 1 b.

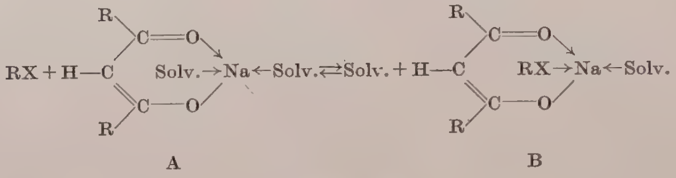
With or without the formation of a covalent compound, the addition to the sodium ion of both oxygen atoms in the anion of ethyl acetoacetate will be favoured, as this addition will give rise to a six-membered ring and fix all atoms in the anion in the same plane, an arrangement favourable to the resonance of the system. For sterical reasons, and due to the fact that in a covalent alkali compound the new orbitals of the metal ion with the lowest energy are one *s*- and three *p*-orbitals leading to a sp^3 hybridisation of the bonds, a tetrahedral configuration of the metal ion is most likely (35). The alkali compound will then be a resonance hybrid of the formula XI.



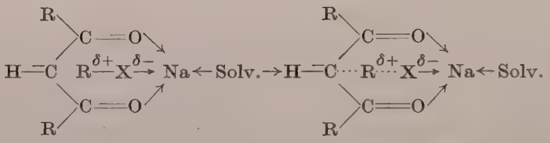
Formula XI

From this it is seen that the compound has a pseudoaromatic structure. The negative charge will not belong to any distinct atom; the electrons will instead be "smeared out" over all the atoms in the ring, and the point of attack of a cationoid reagent will depend on where this reagent finds the highest electron density and where the best interaction, for sterical reasons, can be found between the cationoid reagens and the π electrons of the ring.

4 b. The co-ordination of the halide to the metal ion must be an essential step in the reaction. This can be seen from the fact that the chelate enol form of β-ketoesters and β-diketones are not alkylated by alkyl halides although these compounds have structures very similar to the alkali compounds. The important difference is that the hydrogen ion cannot hold more than two groups, whereas alkali ions can. Whether this co-ordination of the halide to the metal ion in alcoholic solution takes place by the removal of one mole of alcohol already co-ordinated or by an increase in the co-ordination number, is of little interest, and it may for the sake of simplicity be assumed that a co-ordination number 4 is maintained throughout the reaction (Formula XII). The co-ordination causes the withdrawal of electrons from the halogen atom X in the halide RX. This increases the polarisation of the halide molecule and gives the R group a resulting positive charge. The positive charge attracts the π electrons in the pseudo-aromatic ring, and from sterical considerations the best interaction is at the central carbon atom, as this will give rise to a strainless ring system. This can be illustrated by formula XIII, which also gives the transition state in the C-alkylation. As no similar interaction can be found at the oxygen atoms (which would give rise to 4-membered rings), the mesomeric form with the charge on the C-atom will be stabilized. This accounts for a high value of the fraction X_a/X_b in the treatment given in 3 d–3 f, where it was concluded that the high value for the ratio of C- to O-substitution products obtained in alcoholic solution could only be explained by a high value in X_a/X_b.



Formula XII

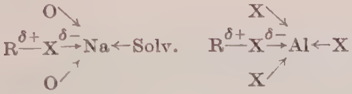


Formula XIII

4 c. A comparison of the alkylation with the aromatic substitution is very interesting, and will be resorted to on several occasions in this paper. Although the aromatic substitution is not clear in every detail, the following seems to be the most important facts for present purposes.

The common aromatic substitution nitration, bromination, and the Friedel-Crafts reaction result from the action of a cationoid reagent. The reaction takes place in two steps, the first of which is the addition of the cation to the aromatic compound, leading to a breakdown of the aromatic electron system (36). This step is the rate-determining one in the case of the nitration of many simple compounds, and for the brominations as well (37). The second step involves the removal of the hydrogen ion from the ring, and is the rate-determining one for the sulphonation (37).

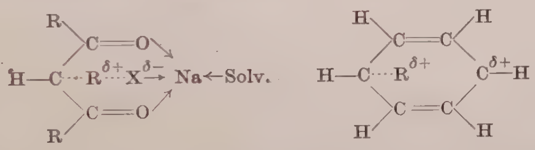
There are exactly corresponding steps in the alkylation reaction. The first step, not mentioned above, the co-ordination of the halide to the metal ion, corresponds to the formation of the active cation in the aromatic substitution, *e.g.* the formation of an alkyl cation from an alkyl halide and aluminium chloride (Formula XIV). The



$$\mathrm{R}\overset{\delta+}{-}\mathrm{X}\overset{\delta-}{\rightarrow}\underset{\mathrm{O}\nearrow}{\overset{\mathrm{O}\searrow}{\mathrm{Na}}}\leftarrow\mathrm{Solv.} \qquad \mathrm{R}\overset{\delta+}{-}\mathrm{X}\overset{\delta-}{\rightarrow}\underset{\mathrm{X}\nearrow}{\overset{\mathrm{X}\searrow}{\mathrm{Al}}}\leftarrow\mathrm{X}$$

Formula XIV

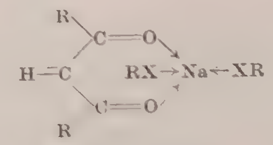
next step, which is the rate-determining one in many substitution reactions, the addition of the cation to the aromatic compound with the breakdown of the aromatic electron system, corresponds closely to the addition of the polarized alkyl group to the C atom in the chelate compound with the breakdown of the pseudo-aromatic electron system (Formula XV). This step is probably the rate-determining one in



Formula XV

this reaction too. The rate of the reaction will therefore depend on the resonance energy in the chelate ring and in the transition state, and this can probably be roughly calculated by a suitable comparison with an aromatic compound. The last step, the removal of the hydrogen ion, is of little importance for the alkylation reaction, since the product obtained is quite stable although it may form a new anion by the removal of the hydrogen ion. This may be of some importance in the alkylation reactions where both mono and disubstitution reactions may take place, but the rate of this step is usually too high to be of any kinetical importance. The alkylation reaction is therefore best compared with simple nitration reactions.

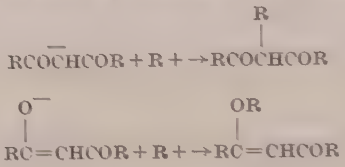
4 d. The alkylation reaction in an ether or benzene suspension may be quite different from that in an alcoholic solution. In an alcoholic solution the alkali metal ion has a high co-ordination number (probably 4 or 6), but in an ether or a benzene suspension the co-ordination number of the alkali ion cannot exceed 2 if no other polar reagent is added. As demonstrated in 1b, these alkali derivatives are usually salts or 2-covalent compounds, which are often readily transformed into more stable salts *e.g.* on heating. On the addition of a polar reagent to these salts or 2-covalent compounds, they aquire the more stable co-ordination numbers 4 or 6, and in so doing they are often transformed into 4-covalent compounds. If a halide is added to a suspension of an alkali derivative of ethyl acetoacetate or related compounds, the first step in the reaction must be a co-ordination of the halide to the alkali ion. If the halide is not highly polarized (i.e. not very reactive) a compound with the co-ordination number 4 is formed (Formula XVI), which then takes part in the alkyla-



Formula XVI

tion reaction in much the same way as the related compound does in an alcoholi solution. C-alkylation compounds are thus formed.

If the halide is very highly polarized (very reactive), the co-ordination to the metal ion will increase the polarisation to such an extent that the halide (usually a acid chloride) splits into ions. The acyl cation so formed will then react with the anio of the alkali compound yielding O- or C-acylation products, according to the electro distribution in this mesomeric anion (Formula XVII). Since in this case no stabiliza



$$\mathrm{RCO\overset{-}{C}HCOR + R^{+} \rightarrow RCO\overset{R}{\overset{|}{C}}HCOR}$$

$$\mathrm{R\overset{O^{-}}{\overset{|}{C}}{=}CHCOR + R^{+} \rightarrow R\overset{OR}{\overset{|}{C}}{=}CHCOR}$$

Formula XVII

tion of one special form of the anion is possible, the reaction with a very reactiv halide such as HCl will indicate the charge distribution in this anion. With halide of reactivities between those of the alkyl halides and that of HCl both this ioni reaction and the reaction involving 4-co-ordination compounds will take place giving increasing amounts of C-reaction products with decreasing reactivity.

A more exact prediction of the amounts of the O- and C-substitution may be mad from the following consideration. The first step in the ionic reaction mentioned abov is a dissociation of the molecule RX into R^+ and X^-. The second step is the reactio $R^+ + A^- \rightarrow RA$. This reaction must be of S_1 type. On the other hand, the reactio involving 4 co-ordinative compounds must be of S_2 type.

4 c. The factors determining the extent of the S_1 and the S_2 mechanisms have bee examined by DEWAR (38). For the effects of groups in R we have the followin facts: $+$I groups (electron attracting) accelerate S_2, retard S_1, while $-$I group (electron repelling) retards S_2, accelerate S_1. $+$E substituents accelerate S_2 bu affect S_1 little, while $-$E substituents accelerate S_1 but affect S_2 little. Therefor substituents in R will favour one of the two mechanisms at the expense of the othe This provides a good possibility for testing the theory.

Exact information about the ratios C- to O-reaction products are very scarce but the few examples which are well known are in good agreement with the theor given.

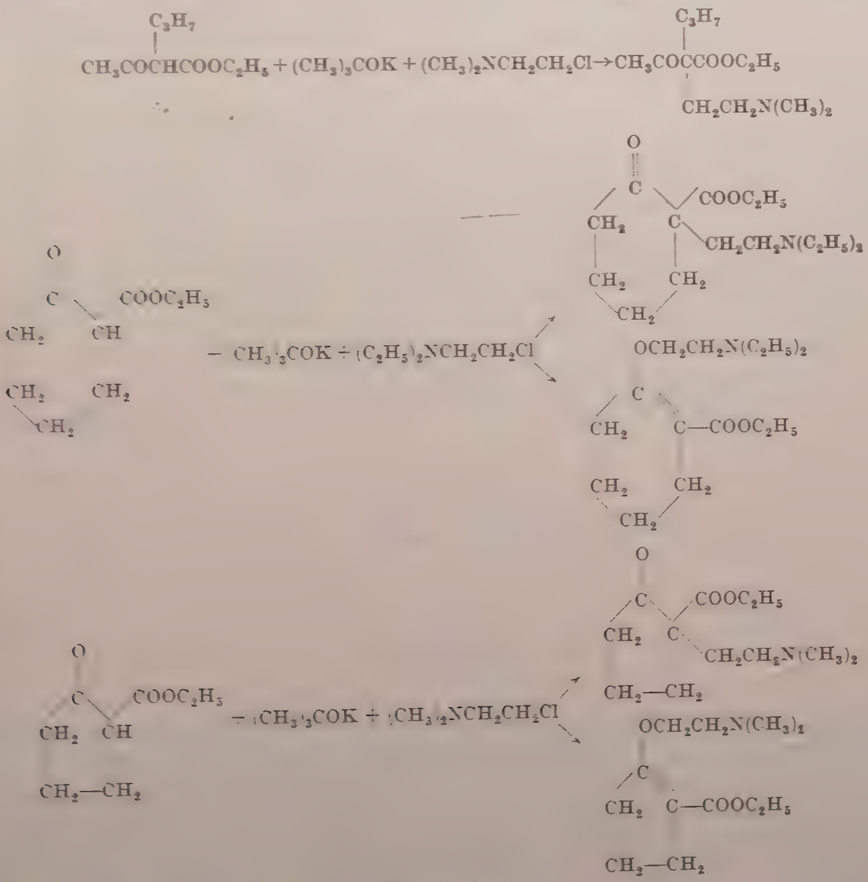
Alkoxy groups are powerful $-$E substituents and favour the S_1 mechanism ,i.e the O-substitution. In fact, alkyl halides give exclusively C-alkyl compounds wit sodium ethyl acetoacetate in an ether suspension, whereas α-chlorodimethyl ethe gives O-alkylation compounds (39), and ethyl chloroformate gives much more O-acy compounds than acetyl chloride under identical conditions.

Table II

The alkylation of some β-ketoesters with β-dialkylaminoethyl chlorides.

Reagents	Method	Yields %		
		total	C	O
I III a	K t-butoxide, t-butanol, reflux 3 hours	49	100	0
II a III a	» » » 6 »	60	19	72
II a III a	» » room temp 67 hours	40	26	66
II a III a	Na sand , toluene , reflux 6 hours	42	42	42
II a III a	Na t-butoxide, t-butanol, » 6 »	70	22	68
II a III b	Na sand , toluene , » 6 »	71	34	51
II b III b	NaH , benzene , » 6 »	50	24	61
II b III b	K t-butoxide , t-butanol, » 3 »	63	22	61
II b III b	K t-butoxide , t-butanol, room temp. 4 hours	71	22	56

I Ethyl α-acetylvalerate, II a 2-carbethoxycyclohexanone, II b 2-carbethoxycyclopentanone, III a β-dimethylaminoethyl chloride, III b β-diethylaminoethyl chloride.



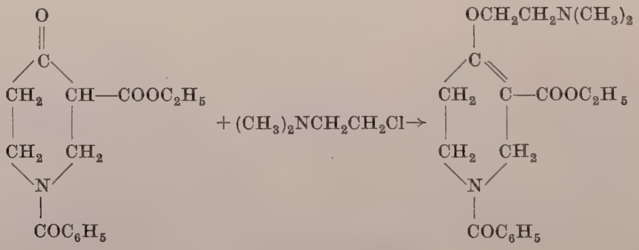
$$CH_3COCH(C_3H_7)COOC_2H_5 + (CH_3)_3COK + (CH_3)_2NCH_2CH_2Cl \rightarrow CH_3COC(C_3H_7)(CH_2CH_2N(CH_3)_2)COOC_2H_5$$

Formula XVIII

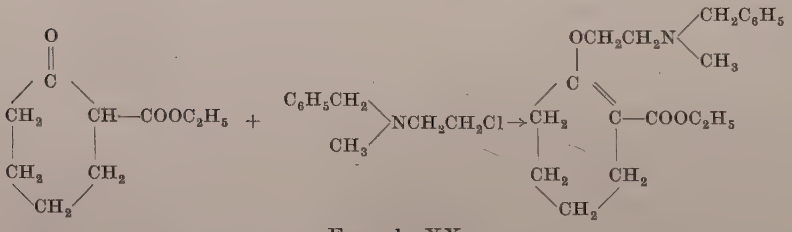
It must be remembered that in testing the theory just given, hetero atoms such as nitrogen and oxygen must not be present in other positions than on the carbon atom holding the halogen atom.

4 f. The effect of nitrogen or oxygen atoms attached to the β-carbon atom of the halide has been studied by many investigators, but no acceptable theory has been given for the "anomalous" reactions obtained.

β-Dialkylaminoethyl chlorides have been reported to give a high proportion of O-alkylation products. The experiments by RHOADS, REYNOLDS and RAULINS (32) given in Table II provide some interesting facts. Open-chain β-ketoesters obviously give no O-alkylation products with these reagents, whereas ringclosed β-ketoesters give good yields (Formula XVIII). The fact that carbethoxycyclopentanone and carbethoxycyclohexanone give the same proportions of C- and O-substitution products indicates that this proportion is not affected by the acidity and enol content of the compound, as these properties are very different for the two compounds mentioned (40). The effect is probably explicable sterically, as is confirmed by the fact that 1-benzoyl-3-carbethoxy-4-piperidone gives exclusivly O-alkylation products with dialkylaminoethyl chlorides (41) (Formula XIX), and carbethoxycyclohexanone only O-alkylation products with benzylmethylaminoethyl chloride (31) (Formula XX).

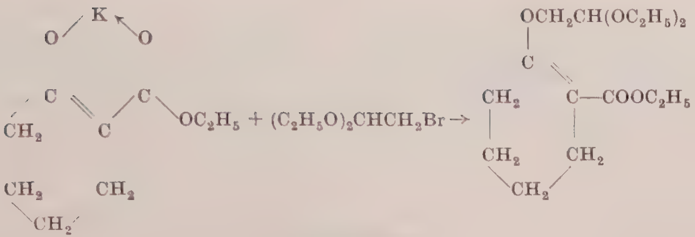


Formula XIX



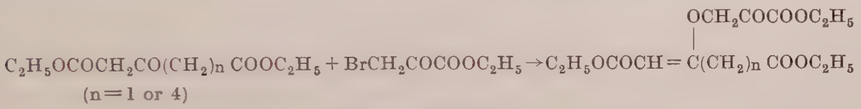
Formula XX

However it cannot only be a question of sterical effects, because branched chain alkyl halides do not give O-alkylation products. It cannot be the rather high reactivity of the halide in this case which is responsible for the "anomalous" reaction, as even such an unreactive halide as bromoacetaldehyde diethylacetal gives O-alkylation of the potassium derivative of carbethoxycyclohexanone (31) (Formula XXI). (This alkali compound is of the 2-covalent type since it is readily soluble in toluene and xylene.)



Formula XXI

Lastly the O-alkylation cannot depend on a preferential S_1 mechanism, as ethyl cetonedicarboxylate and ethyl β-ketosuberate give O-alkylation products with thyl bromopyruvate (42) (Formula XXII). In this compound the $COCOOC_2H_5$ roup is a powerful $+E$ group, which will greatly favour the S_2 reaction.

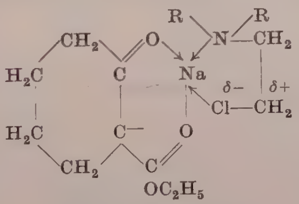


$$C_2H_5OCOCH_2CO(CH_2)_n\,COOC_2H_5 + BrCH_2COCOOC_2H_5 \rightarrow C_2H_5OCOCH{=}\overset{OCH_2COCOOC_2H_5}{\overset{|}{C}}(CH_2)_n\,COOC_2H_5$$

$(n = 1 \text{ or } 4)$

Formula XXII

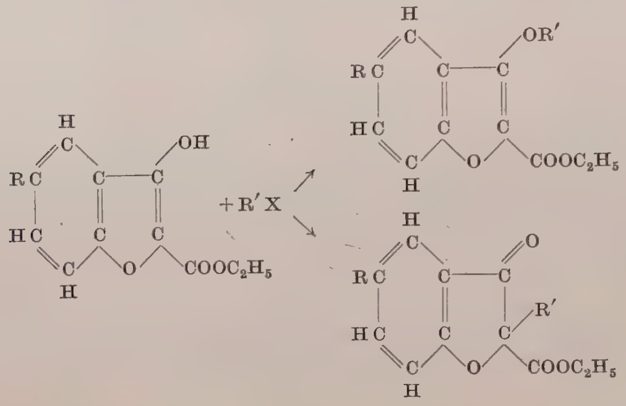
The explanation must depend on some factor common to these three halides. his factor must be that all of them have a polar group on the β-carbon atom. If ow the reaction of β-dialkylaminoethyl chlorides with the alkali compound of a -ketoester is considered, the first step in the reaction must as before be the co-rdination of the halide to the alkali ion. The important difference from the simple lkyl halides is that the nitrogen atom co-ordinates first, which is easily understood n view of the high co-ordination tendency of the amines. However, this co-ordination will not affect the polarisation of the C–X bond very much, though it will give the alogen atom X a favoured position for co-ordination with the same metal ion with he production of a five-membered ring. With carbethoxycyclohexanone the resulting compound will have the structure XXIII. With a tetrahedral configuration



Formula XXIII

f the sodium ion the $\bar{C}$ and the $C^{\delta+}$ atoms will be far away from each other and he alkylation cannot take place within the complex. Instead one mole of the complex must alkylate a second one. For such a reaction the steric effects must be very mportant, and it can be seen from models that the carbon atom is much more free for alkylation in open chained β-ketoesters than in carbethoxycyclohexanone and related cyclic compounds. The experimental facts are however too few to justify more exact conclusions.

4 g. Conjugation of the chelate ring with aromatic rings provides several interesting examples. A good example of this can be obtained from VON AUWERS' investigation on the alkylation of certain derivatives of coumaranone and related substances. The derivatives XXIV A and B of oxy-coumarilic acid were alkylated using different alkyl halides, and the results are given in Table III (43). From this it is obvious that



XXIV A R=H
XXIV B R=CH_3

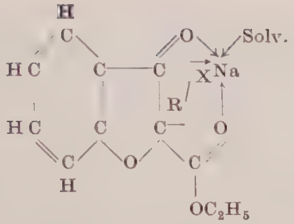
Formula XXIV

Table III

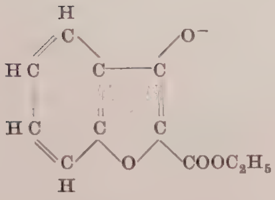
The alkylation of some derivatives of oxy-coumarilic ester.

Alkyl halide used	Compound XXIV A C %	Compound XXIV A O %	Compound XXIV B C %	Compound XXIV B O %
Methyl iodide	53	42.5	54	34
Ethyl iodide	22.5	36	12.5 20.5	46.5 38
Propyl bromide.......	29	56.5	29.5 23	59 47
Allyl bromide	73.5 71.5	— —	55.5 44	— 28
Benzyl chloride.......	68.5	29.5	65 71.5	28 22.5

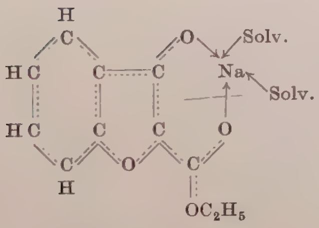
the amount of the C-alkylation product will increase with increasing reactivity o the halide. According to the theory given in 4 b. the C-alkylation will proceed over the complex XXV, whereas the O-alkylation is probably a similar reaction to the alkylation of a phenol, that is, a S_2 reaction between the anion XXVI and the halide RX. The structure of the starting product, the alkali compound, is very probably that of formula XXVII, in equilibrium with the anion XXVI.



Formula XXV



Formula XXVI

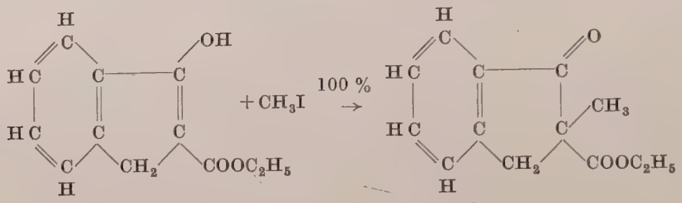


Formula XXVII

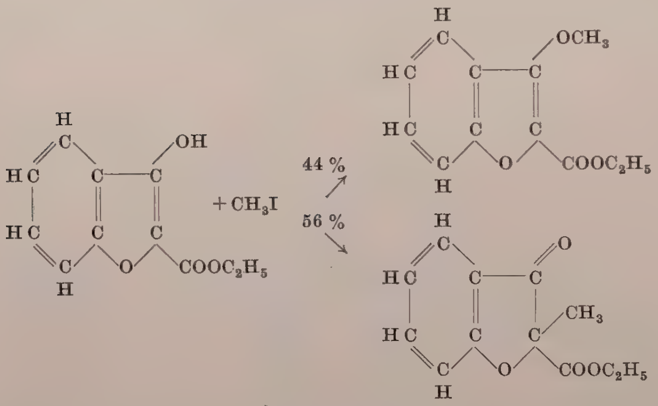
Annulisation of the chelate ring with aromatic rings will affect the stability of the chelate ring system. This has been the subject of many investigations and a review of these has recently been given (44). For the present purpose it is sufficient to state that the annulisation with an aromatic ring will decrease the stability of the chelate ring.

The formation of the transition state in the O-alkylation must involve the loss of the resonance energy of the chelate ring leaving the benzofurane system unaffected, whereas the formation of the transition state of the C-alkylation will decrease the resonance in both the furane ring and the chelate ring, though it is not lost in either ring. It can further be shown that an increasing positive charge on the R group will favour the C-alkylation 5 a. (Within certain limits, of course. Such highly polar halides as the acid chlorides will give rise to just the opposite effect, as mentioned in 4 d.)

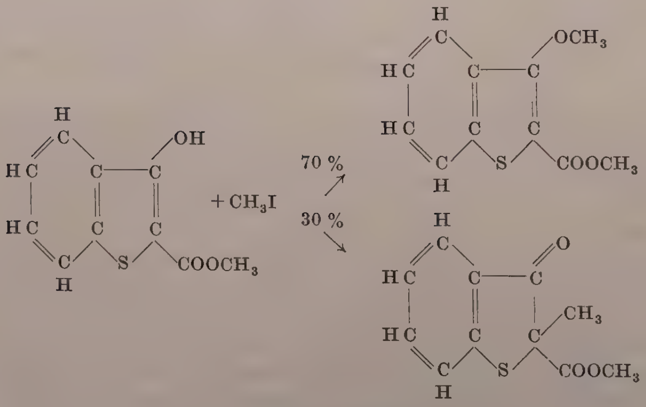
From what has been said above it is also clear that the extent of the C-substitution will decrease with increasing resonance energy of the aromatic ring system, whereas it will increase with increasing stability of the chelate ring. The former effect is easily seen from the data obtained on the methylation of compounds closely related to the oxy-coumarilic ester, such as the corresponding thio derivative and 2-carbethoxy-1-hydrindone. The hydrindone derivative gives only the C-alkylation compound (Formula XXVIII) (45), the benzofurane derivative equal parts of O- and C-methyl compounds (Formula XXIX), and the thionaphthene derivative 5 parts of the O- and 2 parts of the C-methyl derivative (Formula XXX) (46). This corresponds to increasing resonance energies in the order indene < benzofurane < < thionaphthene, which is in good agreement with the well known order.



Formula XXVIII

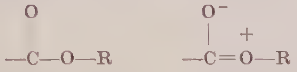


Formula XXIX



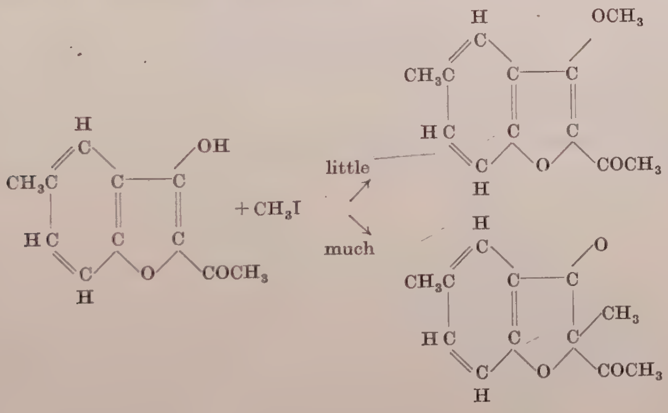
Formula XXX

The stability of the copper chelate of acetylacetone is much greater than that of ethyl acetoacetate. The explanation is (44) that in the case of ethyl acetoacetate, the participation by the carbonyl groups in the very strong ester resonance of the type



$$\overset{\text{O}}{\overset{\|}{—\text{C}}}—\text{O}—\text{R} \qquad \overset{\text{O}^-}{\overset{|}{—\text{C}}}\overset{+}{=}\text{O}—\text{R}$$

greatly interferes with the chelate resonance, thus accounting for the very large decrease in the stability of the chelate. This shows the great importance of the resonance effect as a factor in chelate bond strength since the ester resonance would normally tend to strengthen the bond by increasing the negative charge on the oxygen of the O-Cu bond. This tendency is far outweighed by the effect of weakening the resonance of the chelate ring. It can then be expected that the replacement of the carbethoxy group in oxy-coumarilic ester by an acetyl group will increase the resonance and hence the stability of the chelate ring in the sodium compound, with the effect that more C-alkylation product will be obtained. This is in good agreement with the experimental fact (Formula XXXI) (46). This is a strong evidence for the chelate mechanism, as an ionic mechanism would give rise to just the opposite effect.

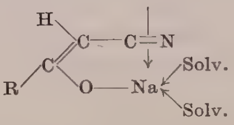


Formula XXXI

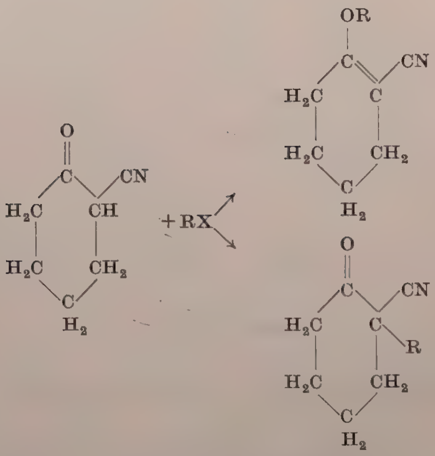
A similar effect can be obtained if the carbethoxy group is replaced by a cyano group.

4 h. *The structure of the alkali derivative of β-ketonitriles* has not been examined very closely and they are usually considered to be salts. They are not always salts, however, since many covalent alkali compounds of cyanoacetic ester and related compounds have been described, and in fact a 2-covalent potassium derivative of ethyl cyanoacetate is mentioned in 1 b., where it was also mentioned that the corresponding sodium compound, although a salt in the solid state, is transformed to a covalent sodium compound on the addition of an excess of ethyl cyanoacetate in benzene solution. The structures of the alkali derivatives of β-ketonitriles and related compounds are therefore very similar to those of β-ketoesters. This may appear rather curious at first sight, since the atomic grouping C–C≡N present in

these compounds must be linear and if the lone electron pair of the nitrogen atom is involved in the co-ordination the metal ion must lie on the same line, i.e. 4 atoms in the six-membered ring must lie in a line which is not possible. However, it is not necessary that the lone electron pair of the nitrogen atom represents the co-ordinated electrons, because the π electrons of multiple bonds are also available for co-ordination. (The π complexes which are formed by such co-ordination have attracted a great interest in the modern theoretical organic chemistry (47).) A concrete example of this possibility is provided by ethylene platinous chloride $(PtCl_2C_2H_4)_2$ in which the π electrons of the ethylene double bond take part in the co-ordination (48). The alkali derivatives of β-ketonitriles would thus have the structure XXXII in alcoholic solution. Such a ring system is strainless but it seems rather probable that the stability will be much less than that of an alkali derivative of a β-ketoester. This will appear in a greater tendency to form O-alkyl compounds. This can readily be seen from the fact that α-cyanocyclohexanone gives a high yield of O-alkylation products with the simple alkyl halides (Formula XXXIII). The effect of increasing polarisation is very clearly demonstrated by Table IV taken from a careful study by von Auwers (49) and Wiegand (50). It should be mentioned that α-carbethoxycyclohexanone gives only C-alkylation compounds under the same conditions (Formula XXXIV). It is also very interesting that α-cyanocyclopentanone gives only C-alkylation products (51) (Formula XXXV). This fact can be explained if the properties of cyclohexanone and cyclopentanone derivatives are compared. α-carbethoxycyclohexanone is very highly enolised, whereas the corresponding α-carbethoxycyclo-



Formula XXXII

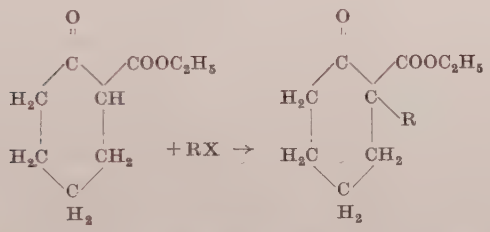


Formula XXXIII

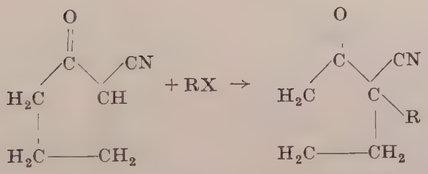
Table IV

The alkylation of α-cyanocyclohexanone.

Alkyl halide	C %		O %	
Methyl iodide	87	82	13	18
Methyl bromide	—	81	—	19
Ethyl iodide	61	65	39	35
Ethyl bromide		39		61
n-Propyl iodide	53	59	47	41
n-Propyl bromide	34	31	66	69
iso-Propyl iodide	21		79	
Benzyl bromide	61	88	39	12
Benzyl chloride	55		45	
Allyl bromide		96		4



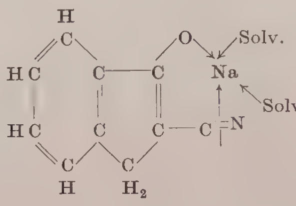
Formula XXXIV



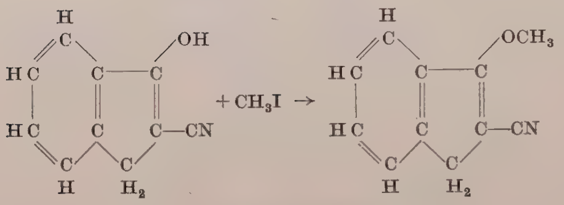
Formula XXXV

pentanone is only 4 % enolised. This must be due to sterical effects, and demonstrates that a double bond is more easily introduced into a six-membered ring than into a five-membered one. As the transition state in the O-alkylation reaction must have a double bond in the ring, it is much more easily obtained in the case of α-cyanocyclohexanone than in that of α-cyanocyclopentanone. The conclusion drawn is that a much higher yield of O-alkylation compounds is obtained with the former compound than with the latter which is in agreement with the experimental facts.

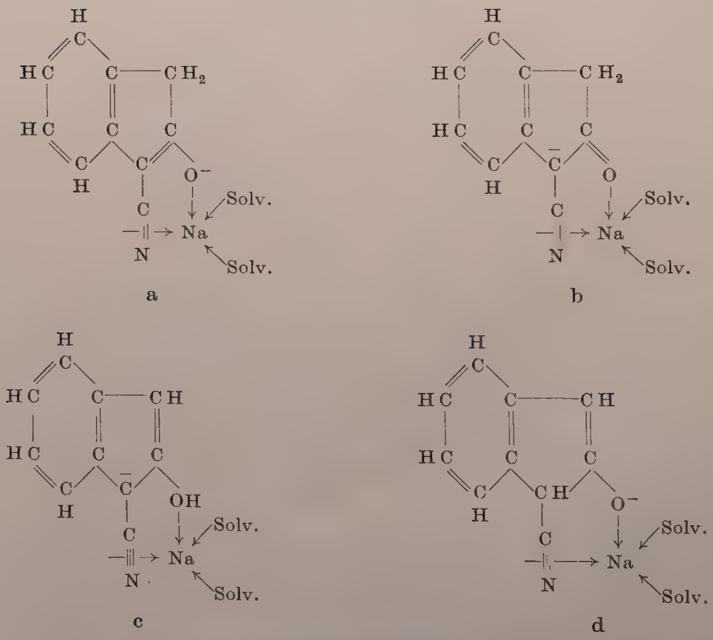
4 i. The alkylation of isomeric cyanohydrindones provides several interesting points. In 4 g it was demonstrated that the resonance in the indene ring system is not high enough to give rise to O-alkylation of 2-carbethoxy-1-hydrindone. In the alkali compound of 2-cyano-1-hydrindone (XXXVI), the stability of the chelate ring is sufficiently low to make a partial loss of the resonance energy of the indene ring system much more important than the loss of the resonance of the chelate ring system. 2-cyano-1-hydrindone is reported to give the O-methylation product if the sodium or potassium compound is treated with methyl iodide in methanol (51) (Formula XXXVII). In the isomeric compound 1-cyano-2-hydrindone we have a somewhat different effect, the structure of the alkali compound of this substance is given in formula XXXVIII, in which some of the more important forms of the anion are given. Form XXXVIII c is a very probable one, it gives rise to the very stable



Formula XXXVI

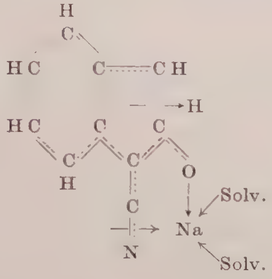


Formula XXXVII

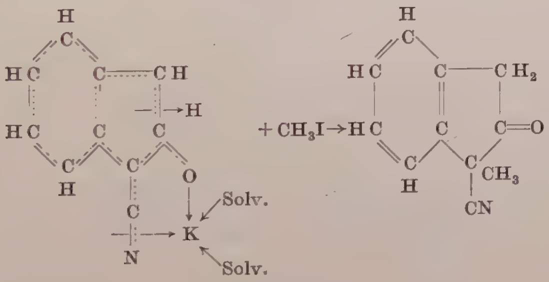


Formula XXXVIII

pentadiene anion ring system. Not all of these forms are mesomeric, since some differ in the position of a hydrogen atom, but a comparison with aromatic compounds differing only in the position of a hydrogen atom will indicate that the activating energy involved in transforming the compounds into each other is very small. This can be expressed by formula XXXIX (52). These facts mean that the chelate ring is highly stabilized, and the energy required to break it is again comparable with that necessary to break this and the aromatic system partly, as is necessary for the transition state in C-alkylation. The result is that more C-alkylation products should be obtained from 1-cyano-2-hydrindone than from the isomeric compound 2-cyano-1-hydrindone. This agrees well with the experimental fact, for it has been reported that the potassium compound of 1-cyano-2-hydrindone will give only the C-methylation product when treated with methyl iodide in methanol (Formula XL) (53). It is also reported, however, that the same compound when treated with ethyl iodide in ethanol gives only the O-ethyl compound. This difference between methyl iodide and ethyl iodide is somewhat greater than might be expected, and although the fact that different solvents were used may explain it to some extent, it seems very probable that the products mentioned were not the only products formed in the reaction but instead the only products isolated from the reaction mixture. However, the fact that a C-methylation product was obtained is firmly established.

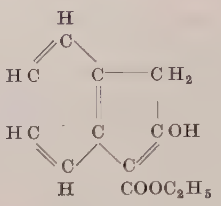


Formula XXXIX

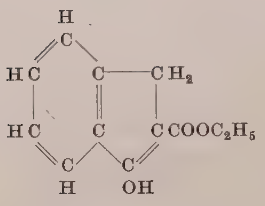


Formula XL

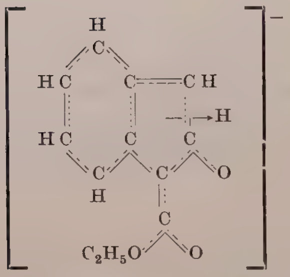
Evidence for the structure given in formula XXXIX can be obtained from the acidity of the isomeric carbethoxyhydrindones (54). The 1-carbethoxy-2-hydrindone (XLI) is a rather strong acid which can be titrated with standard alkali to give an



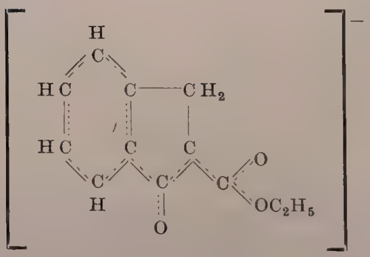
Formula XLI



Formula XLII



Formula XLIII



Formula XLIV

exact equivalent weight for the compound. The 2-carbethoxy-1-hydrindone (XLII) i not such a strong acid and cannot be titrated with standard alkali. This indicate a much higher resonance energy for the anion of the 1-carbethoxy compound (XLIII than for that of the isomeric compound (XLIV), which is in good agreement wit the structure given.

V. Use of the theory to predict relative alkylation rates

In 4 c the close similarity of the alkylation reaction of metallic derivatives of tautomeric substances to that of nitrations of aromatic compounds was pointed out. This analogy will now be used to calculate relative alkylation rates.

5 a. The effect of the polarisation of the halide on the reaction has been pointed out in the alkylation of α-cyanocyclohexanone, and has been observed in many reactions. The effects will now be considered more closely. The first step in the C-alkylation reaction is a co-ordination of the halide to the alkali ion. This has been illustrated by formula XII. With the symbols used there, the reaction can also be formulated as $A + RX \rightleftharpoons B + Solv.$ The concentration of the halide-containing complex B will thus depend on the relative co-ordination properties of the molecules RX and Solv. If the solvent is the same in reactions with different halides, the concentration of B will only depend on the co-ordination properties of RX. (The concentrations of the starting products are of course taken to be constant.) Furthermore if the X atom is the same in the different halogenides, the polarisation of the halide is the only variable factor that can affect the concentration of B. The co-ordination of the X atom to the metal atom will then increase with increasing negative charge on the X atom. It will also increase with increasing positive charge on the R atom as this positive charge can conjugate with the π electrons in the ring and thus give rise to a higher resonance. The resultant effect will be that an increasing polarisation of the RX molecule will increase the co-ordination properties of this molecule, and therefore increase the concentration of B. The next step in the C-alkylation reaction is a simple S_2 reaction. Although this reaction takes place within the molecule, it is probable that it is very similar to a reaction taking place between different molecules such as ether formation from alcoholates and an alkyl halide. The influence of the different R groups should therefore be the same or nearly the same for these two reactions in this step of the reaction. That is, increasing polarisation of the halide will increase the rate of both types of reactions, but since it will also increase the concentration of B in the C-alkylation reaction, the net effect will be that the rate of this reaction is increased much more than the rate of the reaction between alkyl halides and alcoholates. This is clearly demonstrated by the values for the relative rates of some reactions studied by CONRAD and BRÜCKNER (55) (Table V).

Table V

Relative reaction rates of methyl and ethyl iodides with some sodium compounds.

Sodium compound of	Methyliodide	Ethyl iodide
Phenol	5.04	1
Ethyl alcohol	12.66	1
Ethyl acetoacetate	27.8	1
Ethyl ethylacetoacetate	23.6	1
Ethyl heptylacetoacetate	26.1	1
Ethyl allylacetoacetate	24.5	1
Ethyl benzoylacetate	23.6	1

This different influence of substituents on the C-alkylation reaction and the ether formation may explain the different proportion of C- and O-products when different alkyl halides are used. Since the rate of the C-alkylation reaction is increased more by increasing polarisation of the halide than is the O-alkylation reaction, and since this and the ether formation reactions are similar reactions, the effect will be that more C-alkyl products are obtained with more polar halides than with less polar ones. This effect is clearly demonstrated by the values given in Tables III and IV.

5 b. The effect of substituents in the chelate ring should be very similar to the corresponding effect of substituents on the aromatic substitution, as the transition states are very similar. However, it may be somewhat complicated by the effects of the same substituents on the stability of the chelate ring. From the fact that the alkali derivatives of the simple β-ketoesters and β-diketones will give only C-alkylation products, it can be assumed that the alkali compounds of these substances are present largely as co-ordination compounds in an alcoholic solution, and the effect of different substituents may be taken to be rather unimportant in this case, since the first effect of the alkyl halide is to replace one mole of alcohol in the complex. This will not affect the resonance in the chelate ring very much, and substituents in the chelate ring will therefore not affect the equilibrium $A + RX \rightleftharpoons B + \text{Solv.}$ very much. So this effect will be neglected in the following considerations, but it is very important to remember this limitation in using the rules derived.

For the case of simplicity, the carbon atom at which the substitution takes place will be called the α-carbon and the other two carbon atoms in the ring the β- and β'-carbon. As the alkylation comprises the reaction of a cationoid reagent with a pseudo-aromatic ring system, the effect of substituents at the β-carbons will be the same as that of substituents at the ortho carbons of a benzene ring in the aromatic substitution. For the more common substituents at this atom, the relative reaction rates are $COOC_2H_5 < CH_3 < OC_2H_5$. This explains the well known order of reaction rates diketones < ketoesters < malonic esters, and ethoxalyl esters < ketoesters, but more exact data are still lacking.

The effect of substituents on the α-carbon atom must of course be considered from another point of view. In the C-alkylation the complex with the charge on the α-carbon atom corresponds to the anion A^- in the reaction $A^- + RX \rightleftharpoons AR + X^-$. The reaction rate will increase with the reactivity of A^-. As this will increase with increasing negative charge on this atom, it may be concluded that $-I$ groups (alkyl groups) on the α-carbon atom will increase the reaction rate, whereas $+I$ groups (e.g. a phenyl group) will decrease the rate. It must be remembered, however, that since the reaction takes place at the α-carbon atom a substituent on this atom will give rise to a very pronounced steric effect. This is clearly demonstrated by the values of the relative alkylation rates obtained by CONRAD and BRÜCKNER (56) (Table VI), and by the fact that diethyl phenylmalonate is alkylated much more slowly than diethyl malonate (57).

That the steric effects are often very important can be seen from the fact that diethyl *sec*-butylmalonate is rather difficult to alkylate (58), and that diethyl *tert*-butylmalonate is not alkylated by ethyl chloroacetate in an alcoholic solution of sodium ethoxide. (59).

The fact that the alkylation rate of an ethyl alkylacetoacetate is higher than that of ethyl acetoacetate cannot be taken to indicate that the disubstitution product should be the main product in an alkylation of ethyl acetoacetate, for it must be

Table VI

Relative reaction rates of the alkylation of some β-ketoesters in alcohol solution.

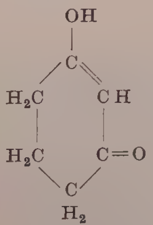
Sodium compound of	Methyl iodide	Ethyl iodide	Propyl iodide
Ethyl acetoacetate	100	3.60	1.30
Ethyl methylacetoacetate	–	12.8	–
Ethyl ethylacetoacetate	291	12.3	5.48
Ethyl heptylacetoacetate	273	10.45	–
Ethyl allylacetoacetate	174	7.10	–
Ethyl benzylacetoacetate	–	5.75	–
Ethyl benzoylacetate	61.6	2.62	–

remembered that it is the alkali derivative of the β-ketoester that is alkylated. The concentration of this will depend on the acid strength of the β-ketoester, which is lowered by the same factors that raise the reaction rates, except that steric effects are operative mainly in the alkylation reaction. The net effect will be that the amounts of the dialkylation product will decrease with the length and branching of the alkyl chain in the alkyl halide used. This is in good agreement with experimental results well known to most organic chemists.

VI. The alkylation mechanism for metallic compounds of β-ketoesters and related substances which are sterically incapable of forming simple chelate compounds

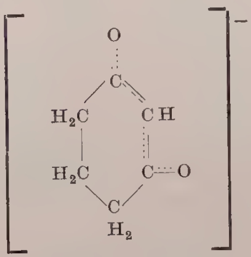
Up to now only compounds whose structure permits a chelate form have been considered. There are, however, many examples of compounds which for sterical reasons cannot have alkali derivatives of a chelate type, but which nevertheless give C-alkylation products. Such a substance is dihydroresorcinol, and as this substance has been rather carefully investigated it is taken as the prototype for substances of this class.

6 a. *Dihydroresorcinol* is almost entirely in the enol-form in water solution (60). It is readily soluble in water and alcohol, but sparingly soluble in ether and ligroine (61), and is a rather strong acid $K_{25} = 5.5 \cdot 10^{-6}$ (62). All this clearly indicates that the compound has a free hydroxyl group, and sterical reasons make the existence of the usual chelate ring of the enol-form impossible. The compound therefore has the formula XLV.

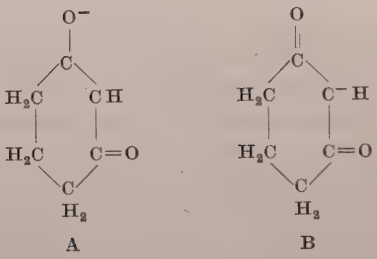


Formula XLV

The alkali derivatives of dihydroresorcinol are typical salts, and exist as such in solutions. The anion of such a salt is a resonance hybrid of structure XLVI, and it is rather well approximated by the structure XLVII A with the negative charge on one of the oxygen atoms. In the reactions with active cations, the reaction products will have structures derived from this, but it must be remembered that the charge distribution in the anion can be readily displaced towards the other main structure XLVII B, with the negative charge on the carbon atom, by reagents which attract electrons in this position.



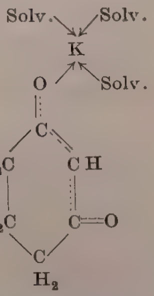
Formula XLVI



A B

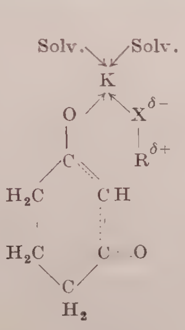
Formula XLVII

In 4 a it was pointed out that all ions are solvated in solution, and that a cation is never far away from a negative charge. If the reasonable assumption is made that the alkali ion is tetrahedrally surrounded, the structure of the alkali compound in solution will approximate to that given in XLVIII, where it is surrounded by thre

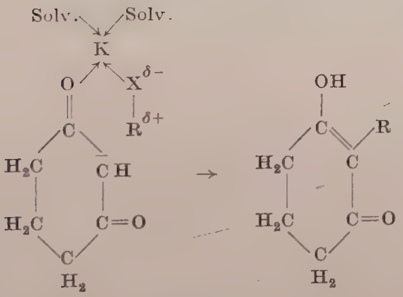


Formula XLVIII

molecules of the solvent and one of the mesomeric anion with an oxygen atom close to the alkali ion. In this structure, one or more of the solvent groups may be replaced by other groups which are also able to co-ordinate with the alkali ion. If the alkali ion is the same the statistical amount of alkali ions surrounded by two moles of the solvent and one mole of a halide (Structure XLIX) will increase with increasing co-ordination tendency of the halide or a decreasing one of the solvent, and will increase with increasing concentration of the halide but decrease with increasing concentration of the solvent. If a compound of structure XLIX is considered, it is readily seen that the halide RX must become highly polarized by the co-ordination with the metal ion, and the R atom will have a positive charge as a result. This attracts an atom with a high electron density, and such an atom can be found in the central carbon atom of the mesomeric anion, as in this case, a strainless six-membered ring is formed. It is obvious that such a structure favours the C-alkylation (Formula L),



Formula XLIX



Formula L

and it is very probable that the C-alkylation product is obtained by this mechanism, and that the O-alkylation product results from a reaction with the anion without this stabilization of the structure with the negative charge on the carbon atom. If this account is correct, the following factors should affect the proportion of the different alkylation products: the concentration of the alkyl halide and the solvent, the co-ordination properties and polarisation of the halide, the co-ordination tendency of the solvent, the alkali ion used, and the structure of the anion. These factors will now be examined.

6 b. The importance of the concentration for the extent of the C-alkylation has not been studied much. From what has been said above, however, it is clear that an increasing concentration of the solvent decreases the extent of the C-alkylation. The same effect can also be obtained by decreasing the concentration of the halide. This means that less C-alkylation products are obtained in a very dilute solution than in a more concentrated one. This is seen to be the case from the results obtained by H. STETTER and W. DIERCHS for the alkylation of dihydroresorcinol (63) (Table VII).

Table VII

The effect of the concentration on the alkylation of the potassium derivative of dihydroresorcinol 0.1 mole with butyl bromide in a methanol solution.

Ml methanol	Time	C-compound	O-compound	Total yield
30	5 hours	18.2 %	42.4 %	60.6 %
50	5 »	17.6 %	36.4 %	53.9 %
200	15 »	6 %	25 %	31 %

6 c. The importance of the co-ordination tendency and polarisation of the halide has been examined by the same authors (63) and many others. As increasing polarisation of the halide will give rise to an increasing positive charge on the R atom, which results in a stronger interaction with the central carbon atom in the anion, and to greater negative charge on the halogen atom, giving rise to a higher co-ordination tendency, the net effect will be that increased polarisation of the halide will favour the C-alkylation reaction. This effect is very often observed, and is clearly demonstrated by the values of H. STETTER and W. DIERICHS for the alkylation of dihydroresorcinol (63) (Table VIII).

Table VIII

The reaction of the potassium derivative of dihydroresorcinol with different alky halides in a methanol solution.

Alkyl iodide	Time	C-compound	O-compound	Total yield
Methyl	45 min.	51.5	—	51.5
Ethyl..................	3 hours	27.2	43	70.2
Propyl	3 »	26.0	32.5	58.5
Butyl..................	3 »	28.4	36.4	64.8
Cetyl	24 »	27.0	51.0	78.0

6 d. The effect of different alkali ions was also studied by the same authors (63), an effect which seemed rather curious to them. The theory given above leads us to expect such an effect.

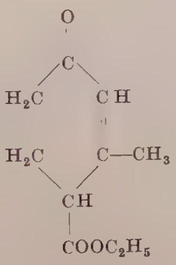
In 1 b. it was demonstrated that the co-ordination tendency of the different alkali ions in forming chelate compounds increased with increasing atomic number of the alkali ion. As the same effect can be expected here increasing amounts of C-alkyla ion should be obtained with increasing atomic number of the alkali atom. This ffect is clearly seen from the investigation of H. STETTER and W. DIERICHS (63) Table IX).

Table IX

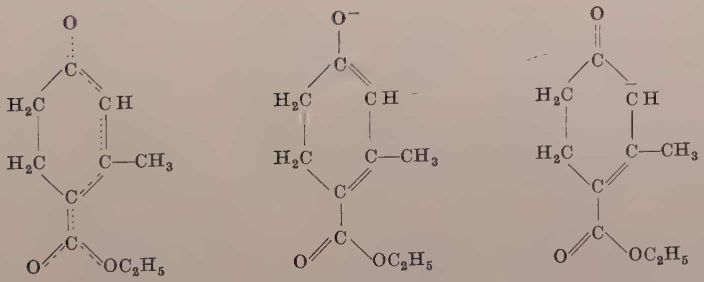
The reaction of different alkali derivatives of dihydroresorcinol with butyl bromide in a methanol solution.

Metal	C-alkylation	O-alkylation	Total yield
Li	6.2 %	21.0 %	27.2 %
Na	15.0 %	36.5 %	51.5 %
K	17.5 %	36.4 %	53.9 %

6 e. The alkylation of Hagemann's ester (LI) is very similar to that of dihydroresorcinol. In the alkali compounds of this ester the conjugation of the double bond and the carbethoxy group with the oxygen atom in the enolised carbonyl group gives rise to a mesomeric anion (LII), which gives rise to O-substitution products in the reactions with active cations (64), indicating that the structure of the anion is rather well approximated by the structure LII A, with the charge on the oxygen atom. However, the charge distribution in this anion is readily displaced in favour of another structure LII B on the addition of an alkyl halide, owing to the formation of a



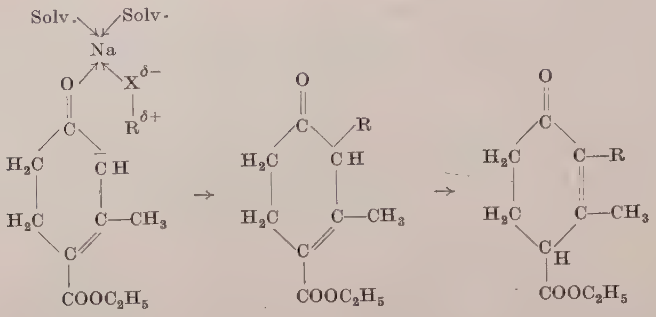
Formula LI



A

B

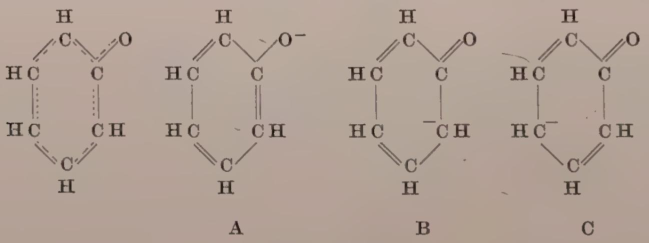
Formula LII



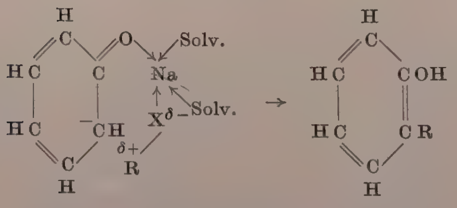
Formula LIII

six-membered ring (Formula LIII) similar to that indicated above for the anion of dihydroresorcinol. This is in good agreement with the experimental facts, as the ester gives C-alkylation products with alkyl halides (65–67). The experimental results are too few, however, to justify any definite conclusions about the relative proportions of the different alkylation products.

6 *f. The alkylation of phenols* has been more thoroughly investigated and illustrates an interesting effect. The phenolate anion is a typical mesomeric anion (Formula LIV) with high anionoid activity on the oxygen atom and in the ortho and para positions, where it is readily substituted under the action of various cationoid reagents. The structure of the anion may be taken to be approximately the same as the structure with the negative charge on the oxygen atom (LIV A). In the reaction with an alkyl halide, formation of a six-membered ring may favour the structure with the negative charge in the ortho position (LIV B) as in the case of dihydroresorcinol. This will give rise to a ring substitution in the o-position relative to the phenol group (Formula LV). This reaction must have a transition state where the resonance energy

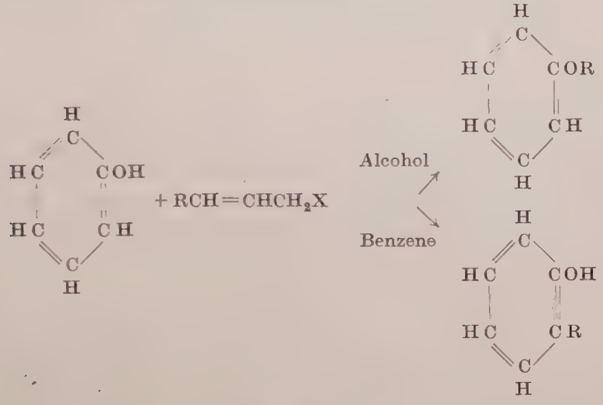


Formula LIV



Formula LV

of the benzene ring is partly lost. This means that the ring substitution is usually not favoured, and that it can be expected only under conditions like those very favourable to the C-alkylation of dihydroresorcinol. In 6 a it was pointed out that a highly reactive alkyl halide in a non-polar solvent greatly favours the C-alkylation reaction. It is actually found that allylic halides give o-allyl phenols when the reaction is performed in a non-polar solvent (68) whereas in other cases only allyl ethers are obtained with simple phenols (Formula LVI).



Formula LVI

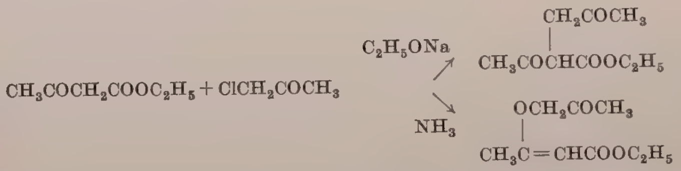
In this connection it may be pointed out that a great many ortho substitutions of aromatic compounds have been explained by a similar chelate mechanism, but we will not go further into this matter, as it does not give any new aspects of interest. Many of the substitutions have been treated by JOHNSON (69), who gives full references.

VII. The alkylation of the copper, silver and ammonium derivatives of β-ketoesters and related substances

7 a. The copper derivatives of β-diketones and β-ketoesters are typical covalent compounds with the very stable co-ordination number 4. As the anions of the β-diketones and β-ketoesters are didentate groups, all four co-ordination places are filled and the molecule has no tendency to co-ordinate other groups. In the alkylation of the alkali derivatives it was pointed out that a co-ordination of the halide to the metal ion was the first step in the reaction. As this step is not possible with the copper derivative, the reaction must follow some other course. MICHAEL (70) has shown that the copper derivatives of ethyl acetoacetate and related compounds are not acylated in the usual way. As the reaction is extremely complicated, and of limited interest in this connection, we will not consider it further. The results obtained by MICHAEL are mentioned as they provide a good illustration of the importance of a preliminary co-ordination to the metal ion. A similar effect has been observed by the author in the kinetic study of the acidolysis of ethyl diacetoacetate by different metallic catalysts (71).

7 b. *The silver derivatives* of acetylacetone and related substances are typical salts, and since the co-ordination number of the silver ion is generally 2 and the configuration linear, the formation of six-membered chelate rings are greatly disfavoured. This means that a stabilization of a form of the anion with the negative charge on the carbon atom cannot take place to any considerable extent. O-alkylation products may therefore be expected even in the reaction with simple alkyl halides such as methyl and ethyl iodide. This has in fact been observed (30, 72) but even here the experimental results are much too scanty to justify any exact calculation of the effect of different substituents on the relative amounts of C- and O-alkylation products.

7 c. *The ammonium derivative* of ethyl acetoacetate must of course be a salt, without any tendency to be transformed to a covalent compound on further co-ordination, because such co-ordination is impossible. In the reactions of ammonium and related compounds we find examples of the reactions of the pure anion. Such reactions have been but little investigated, though available information gives good evidence for the alkylation mechanism put forward in this paper.



$$CH_3COCH_2COOC_2H_5 + ClCH_2COCH_3 \begin{array}{l} \xrightarrow{C_2H_5ONa} CH_3CO\overset{\overset{\displaystyle CH_2COCH_3}{|}}{C}HCOOC_2H_5 \\ \xrightarrow[NH_3]{} CH_3\overset{\overset{\displaystyle OCH_2COCH_3}{|}}{C}{=}CHCOOC_2H_5 \end{array}$$

Formula LVII

The sodium derivative of ethyl acetoacetate gives the expected C-alkyl derivative when treated with chloroacetone in alcohol solutoin, but the ammonium derivative in ether suspension gives the O-alkylation product (73) (Formula LVII). This is in good agreement with the theory given. The fact that β-ketoesters give pure O-acyl derivatives when treated with acid chlorides in a pyridine solution is also just what is to be expected.

SUMMARY

A theory for the alkylation of metallic derivatives of tautomeric substances is given.

The alkali derivatives of tautomeric substances are often typical salts, but some of them are covalent compounds. The salts are often changed into covalent compounds by the addition of a polar compound.

A six-membered ring may be formed on the addition of a halide. The ring contains the halide, the alkali ion and the mesomeric anion. This ring stabilizes the form of the mesomeric anion with the charge on the carbon atom, and thus favours the C-substitution.

The reaction of the mesomeric anion without this stabilization on the other hand, gives O- and C-substitution products according to the charge distribution in this unstabilized anion.

The factors determining the degree of formation of the six-membered ring are examined, and illustrated by a large number of reactions reported in chemical literature. It is demonstrated that many reactions which hitherto have been considered as "anomalous" are readily explained by the theory advanced.

The theory has been used to predict relative reaction rates, and found to be useful in this respect.

The similarity of the alkylation reaction and the cationoid aromatic substitution is pointed out, and it seems that such a comparison might give valuable information about both reactions, as they are both extensively investigated.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, January 1953.

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Incorporation of C^{14}-labeled carbonate and acetate into sea urchin embryos

By Tore Hultin

With 1 figure in the text

It has been shown (7) that the rate of incorporation of N^{15}-ammonium chloride into the pyrimidines and purines of *Paracentrotus* embryos increases rapidly during the early blastula stage. The experiments suggested that this increase coincided with the period of determination of the primary germ layers, and with the exponential respiratory increase, described by Lindahl (12, 13). In the experiments on *Psammechinus* embryos, reported in this paper, the question has been studied more closely, by means of C^{14}-labeled carbonate. The metabolic utilization of 1-C^{14}-acetate in the embryos at different stages, which in certain respects is related to the carbonate metabolism, has also been studied.

Experimental

Egg material: Eggs and embryos of the sea urchin *Psammechinus miliaris* (Gmelin) were used in the experiments. Dilute suspensions of eggs were fertilized and allowed to develop at 20° C in rocking glass trays (11). Equal samples were taken from the homogenous suspensions at certain stages of development. The samples were washed by centrifuging and transferred to fresh sea water containing the isotopic component. Jelly-free eggs were used in the experiments.

Isotope incubations: It has been shown previously (9) that the incorporation of C^{14}-carbonate into sea urchin embryos proceeds more rapidly when the pH of the medium is decreased slightly below that of ordinary sea water. In the present experiments the sea water was adjusted to pH 7.1 and buffered with 0.005 M glycyl-glycine (18).

In order to avoid any considerable dilution of the added isotope by respiratory carbon dioxide emitted from the embryos (cf. 13, 9) the $NaHC^{14}O_3$ used in the experiments had been diluted with carbonate carrier to a final isotope concentration of approximately 1 mC per millimole. The egg samples, taken at different times from the same suspension, were incubated with equal amounts of isotope (approximately 10 μCs in 10 ml of sea water per mg total egg nitrogen). The isotope treatments were carried out in closed glass tubes, which were rocked about a horizontal position.

The experiments, tabulated in Table 2, were performed on the basis of the respiratory measurements of Borei (1). Suspensions of fertilized eggs were divided in 3 equal parts. One part was incubated with the isotope during a period immediately before the steepest rise of the early respiratory increase (2-64-cell stages). The second part was incubated during the whole course of this respiratory increase (32-cell stage-ciliated blastula stage). Finally, the third part was treated with the isotope in the gastrula stage. The samples were incubated with the isotope for exactly 3 ½ hours. Three independent series of experiments were performed, and were subsequently worked up together.

The experiments with C^{14}-acetate were performed in a very similar manner to the carbonate experiments. The samples were allowed to develop for 4 hours in sea water, containing 0.15 µCs 1-C^{14}-acetate[1] (approximately 1.5 mCs per mM) per ml. Approximately 30 ml of sea water containing the isotope were used per mg of total egg nitrogen.

Fractionation of the material: The material was fractionated by a procedure described in detail in a previous paper (7). The samples were extracted repeatedly with cold trichloroacetic acid (TCA) and lipid solvents. The ribonucleic acid was isolated in the form of mononucleotides after fractionated hydrolytic extraction with cold N $HClO_4$ (cf. 14). Finally, the proteins were purified by extraction with 5 % TCA at 90° C (cf. 16).

Because of the very limited amounts of *Psammechinus*-material available, an isotope dilution technique had to be used in certain cases.

In order to remove the TCA, the TCA-extracts were thoroughly treated with ether in a liquid extractor. On determining the ultraviolet absorption of the solutions, an absorption maximum was found at 2 500–2 550 Å. The difference in absorption between 2 480 and 2 800 Å was calculated, and used as an approximate measure of the amounts of free hypoxanthine (7) present in the extracts. An approximately tenfold isotope dilution of the free hypoxanthine was effected by addition of carrier hypoxanthine. The hypoxanthine was isolated by precipitation with $CuSO_4 + NaHSO_3$ as described previously (7). The purified hypoxanthine was combusted, and the specific activity of the carbon was determined.

In the C^{14}-carbonate experiments free amino acids and carbohydrates were isolated from the TCA-extracts. The extracts were acidified with HCl, and Cu-ions were removed by treatment with H_2S. The clear solutions were repeatedly evoporated in vacuo, taken up in small volumes of water and run through a series of 3 coupled ion exchange columns: 1) kation-exchanger (Amberlite IR 120, H^+-form, 25 cm × 0.8 cm^2), 2) anion-exchanger (Amberlite IR 4, CO_3^{--} -form, 15 cm × 0.8 cm^2), 3) kation-exchanger (Amberlite IR 120, 2 cm × 0.5 cm^2). The columns were washed with 250 ml of water. The water-solutions were evaporated in vacuo, and the residues, containing non-polar carbohydrates, mainly glucogen and glucose, were combusted for determination of their specific activities. From the first kation-exchange columns free amino acids were eluted by 200 ml of 4 N HCl. The eluates were evaporated in vacuo, and the amino acids were decarboxylated by ninhydrin treatment (19, cf. 9).

The lipid extracts were evaporated in vacuo, and the residues were dissolved in ether, filtered and again evaporated in vacuo. The lipids were refluxed with 0.1 N alcoholic NaOH for 60 minutes. The hydrolyzates were acidified with HCl and evaporated in vacuo. The fatty acids were extracted with ether, filtered, evaporated and dissolved in dilute NaOH. The fatty acids were precipitated from the alkaline water-solutions by acidification.

The ultraviolet absorption spectra of the $HClO_4$-extracts were determined in a Beckmann-spectrophotometer. The main part of the ribonucleic acid had already been brought into solution by the first two $HClO_4$-extractions (7). These extracts were collected and an approximately tenfold isotope dilution of the labeled nucleotides was effected by addition of calculated amounts of a nucleotide mixture, prepared from commercial yeast nucleic acid. The dilution was performed on the basis of the difference in absorption between 2 600 and 2 900 Å. The mixtures were neutralized with $Ba(OH)_2$ and evaporated in vacuo, and the barium perchlorate was removed by extraction with alcohol-ether (1 : 2). In the C^{14}-carbonate experiments mononucleotides were prepared by chromatography as described previously (7). In the C^{14}-acetate experiments nucleotides were isolated by the method of KERR and SERAIDARIAN (10).

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[1] Kindly supplied by Dr. G. EHRENSVÄRD.

The proteins were hydrolyzed in 10 % HCl for 10 hours at 120° C, and the amino acids were decarboxylated by ninhydrin treatment (19).

Results and discussion

The overall incorporation of C^{14}-carbonate into *Psammechinus* embryos at different stages of development is shown in Table 1. A sharp increase in the rate of isotope incorporation was found during the period of blastula formation. This increase was very pronounced in the TCA-soluble fraction (cf. 9) and coincided closely with the exponential respiratory increase (Table 2).

As is well known, carbon dioxide is directly utilized in the formation of purines (17). In the experiments with C^{14}-carbonate the isotope incorporation into the purines was of especial interest. As is shown in Table 2 the incorporation into free hypoxantine, and into guanine and adenine isolated from ribonucleic acid, was very much intensified during the period of the exponential respiratory increase. These results agreed very well with previous experiments (7), where the incorporation of N^{15}-ammonium chloride into the pyrimidines and purines of *Paracentrotus* embryos was studied. In the present experiments, however, the rate of isotope incorporation decreased somewhat more rapidly during the gastrulation. This may be due to the intensified production of respiratory carbon dioxide in the cells after the mesenchyme blastula stage (cf. 13). Because of the relatively large amounts of isotope carrier used in these experiments, the respiratory carbon dioxide could not influence the isotope level of the medium to any large extent. The formation of intracellular isotope dilution gradients under the influence of the respiratory carbon dioxide must, however, be taken into account. The experiments with C^{14}-acetate will show that respiratory carbon dioxide is actually taken over directly by the purines of the embryos.

The isotope incorporation into the carbohydrate fraction proceeded in a very similar way to the incorporation into the purines (Table 2). It has been shown by GUSTAFSON *et al.* (3, 4) that the mitochondrial activity of sea urchin embryos rapidly increases from the mesenchyme blastula stage. During the course of the

Table 1

Psammechinus embryos at different stages of development kept for 3½ hours in sea water containing $NaHC^{14}O_3$. Specific activity of total C, counts per minute per 15 mg $BaCO_3$ per cm².

Incubation periods, hours after fertilization	Counts/minutes
Unfertilized	90
½– 4 hours	200
3½– 7	560
7 –10½	540
10½–14	530
19 –23	550

Table 2

Incorporation of C^{14} carbonate into *Psammechinus* embryos, studied in relation to the period of exponential respiratory increase (13, 1) Counts per minute per 15 mg $BaCO_3$ per cm^2.

Stages of development	2–64 cell stages	Period of exponential respiratory increase, 32-cell stage-ciliated blastula stage	Gastrula stage
Hours after fertilization	1–4½	4–7½	16½–20
TCA-soluble fraction:			
Total fraction	990	2 600	1 600
Hypoxanthine	5 100	31 000	16 000
Carbohydrate fraction	1 500	6 000	3 500
Amino acid carboxyl-C	460	850	2 800
Lipid fraction:			
Fatty acids	3	5	10
Ribonucleic acid fraction:			
Adenine	500	2 900	2 700
Guanine	610	5 200	2 700
Proteins, carboxyl-C	460	610	1 500

exponential respiratory increase the mitochondrial activity is still relatively low. The rapid incorporation of C^{14}-carbonate into the carbohydrate fraction during this period can therefore not be explained on the basis of an increased mitochondrial activity. It may be due to alterations in the metabolic pattern of the embryos during the determination period, possibly related to the increasing nucleotide metabolism (9, 6). This problem is currently the subject of a more detailed analysis. The isotope incorporation into the amino acids can be more readily interpreted on the mitochondrial basis (cf. 8). In the fatty acids, no significant isotope activity was found.

The sharp increase in the mitochondrial activity from the mesenchyme blastula stage (3, 4) has a very profound influence on the intermediary metabolism of the embryos. This fact was clearly shown by the experiments with 1-C^{14}-acetate (Fig. 1). Before the mesenchyme blastula stage the isotope incorporation into the fatty acids and into the carboxyl groups of the protein amino acids proceeded relatively slowly. During the course of gastrulation, however, a much higher isotope level was attained. In the case of the proteins, the rate of protein rebuilding (5) seemed finally to become a limiting factor.

It has been shown by ELWYN and SPRINSON (2) that acetate is utilized in the synthesis of purines only in the form of respiratory carbon dioxide. As would be expected from this observation, the rate of isotope incorporation into free hypoxanthine and into ribonucleic acid nucleotides increased very conspicuously during the gastrulation (Fig. 1). A smaller, but quite distinct rise in the rate of isotope incorporation occurred during the period of the exponential respir-

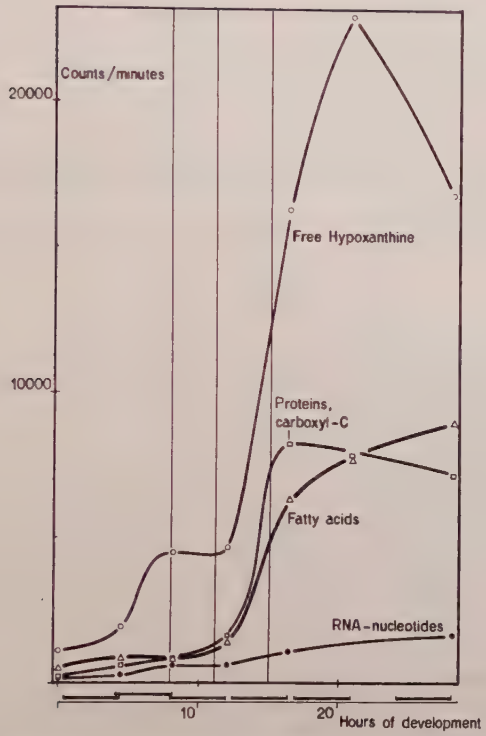

Fig. 1. *Psammechinus* embryos at different stages of development kept for 4 hours in sea water containing 1-C^{14}-acetate. Specific activities (counts per minute per 15 mg $BaCO_3$ per cm²) of fractions, subsequently isolated from the embryos. Vertical lines indicate hatching, appearance of mesenchyme cells and start of invagination. The periods of isotope treatment are indicated by horizontal lines at the bottom of the figure.

atory increase. This rise corresponds to the increased metabolic rate of purines, which in the C^{14}-carbonate experiments was shown to occur during this period (Table 2).

The experiments with 1-C^{14}-acetate indicate that the metabolic utilization of acetate (and probably of higher fatty acids) is quantitatively (cf. 15) not very extensive before the mesenchyme blastula stage. The results are in harmony with the statement of Lindahl (13) that the exponential respiratory increase involves an oxidative breakdown of carbohydrates (cf. 9, 6). The metabolic accessibility of acetate rapidly increases from the mesenchyme blastula stage, simultaneously with the augmented mitocheondrial activity (3, 4).

SUMMARY

C^{14}-carbonate was readily incorporated into the purines of developing sea urchin embryos. The rate of incorporation increased very rapidly during the period of exponential respiratory increase. A similar picture of isotope incorporation was shown by a carbohydrate fraction isolated from the embryos.

The incorporation rate of 1-C^{14}-acetate into the free hypoxanthine and into

ribonucleic acid nucleotides showed a corresponding, but much more limited, rise during the same period. In this case, however, the incorporation intensified rapidly during the mesenchyme blastula stage. At the same time, the utilization of 1-C^{14}-acetate for the formation of protein amino acids and fatty acids increased rapidly. It is known that acetate is utilized in the formation of purines only in the form of respiratory carbon dioxide. The experiments therefore indicate that the metabolic utilization of acetate is highly intensified during the mesenchyme blastula stage.

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The function of magnesium oxide in the acidolysis of diethyl acetylmalonate and ethyl diacetoacetate

By ARNE BRÄNDSTRÖM

With 11 figures in the text

Recently, the present author has described some methods of preparing β-ketoesters (1) and β-diketones (2) as well as some interesting side reactions (3,4) in these syntheses. The starting materials in these reactions, the diethyl acylmalonates, were acidolysed with organic acids using magnesium oxide as a catalyst. In order to find a possible mechanism for these reactions, a kinetic study of the reaction of ethyl diacetoacetate with acetic acid has been undertaken. Ethyl diacetoacetate was used rather than diethyl acetylmalonate to minimize the side reactions, but the results can be applied to diethyl acetylmalonate as the main reactions are of the same type:

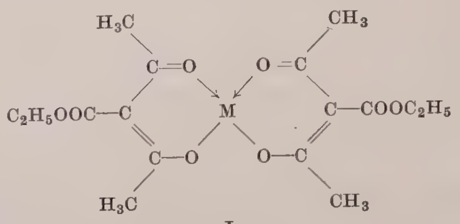
$$CH_3COCH(COOC_2H_5)_2 + CH_3COOH \rightarrow CH_3COCH_2COOC_2H_5 + CH_3COOC_2H_5 + CO_2$$

$$(CH_3CO)_2CHCOOC_2H_5 + CH_3COOH \rightarrow CH_3COCH_2COCH_3 + CH_3COOC_2H_5 + CO_2$$

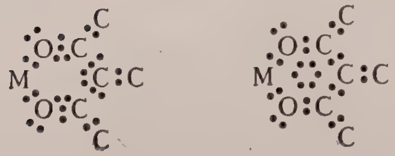
The kinetic study gives the following results:

1. With magnesium oxide as the catalyst, the reaction velocity is proportional to the concentration of the acetic acid and that of ethyl diacetoacetate, and to the square root of the amount of magnesium oxide added.
2. With lithium carbonate as the catalyst the reaction velocity is proportional to the concentration of ethyl diacetoacetate and to the three fourth power of the amount of lithium carbonate added.
3. With zinc acetate as the catalyst, the reaction seems to be the same as that with magnesium oxide, but the reaction velocity is only a sixth of that with equimolar amounts of the latter reagent. With cadmium acetate as the catalyst the reaction is still slower.
4. With boric acid as the catalyst, the reaction is accompanied by a considerable darkening of the product and the values obtained are not very good, but the reaction velocity appears inversely proportional to the second power of the amount of acetylacetone formed in the reaction.
5. With copper acetate, beryllium acetylacetonate, and aluminium acetylacetonate no catalytic effect is observed.

These facts make it probable that the catalytic effects are due to some form of complex formation with the metallic ion. It is well known that β-diketones and related compounds form very stable complexes with most metals. With a divalent



I



II

element ethyl diacetoacetate can form compound I. Owing to the favorable steric relations and resonance effects, these rings would be very stable. The resonance effect in such a ring can be illustrated in a striking way by the electronic formula for the rings with the acidity placed at the carbon atoms. (Formula II.) In this formula the electron sextet would, according to Bamberger's "rule of six", indicate a very high resonance and certain aromatic properties. As an immediate consequence of the resonance, the rings are planar, with the carbethoxyl groups as far as possible from the metal atom. This makes it impossible for the ethyl diacetoacetate group to function as a tridentate group. If the stable coordination maximum for the divalent element is six, as for magnesium, the two vacant coordination places must be filled by coordination of one didentate or two unidentate groups. These groups might be acetic acid or some of the reaction products.

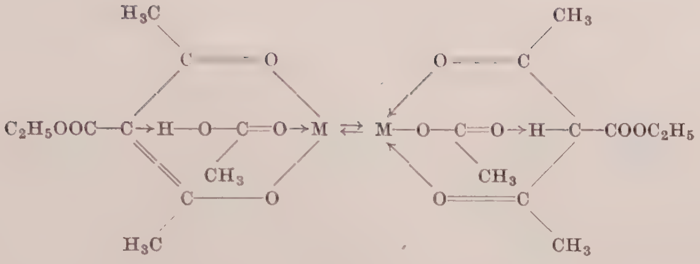
If two moles of acetic acid are coordinated to the magnesium compound of ethyl diacetoacetate, the resulting product is probably the same as if two moles of ethyl diacetoacetate are coordinated to magnesium acetate. Such a state might be represented by formula III. The hydrogen bond in this formula seems quite possible sterically, and the resonance mentioned will make it still more probable.

The hydrogen bond formulation may explain the acidolysis as a α, γ-shift of the acetate ion. (Formula IV.)

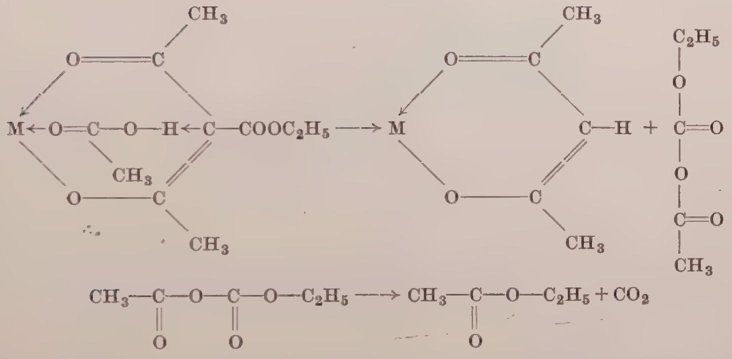
There are also other centers of acceptor activity in the molecule, viz. at the carbon atoms in the other two γ-positions. If an acid with an acyl group different from that of the diethyl acylmalonate (or the ethyl diacetoacetate) is used in the reaction, the shift of the acid anion to one of these γ-positions might lead to the reactions indicated in formula V. The net effect of these reactions will be an acyl exchange. In fact, such an exchange has been reported in the reaction of diethyl acetylmalonate with some acids (3). An experiment with ethyl diacetoacetate and butyric acid shows that an exchange is possible in this reaction too.

It must be pointed out that the acyl exchange reported is by no means a proof of the hydrogen bond formulation as the same result may be obtained by other formulations as well.

Another mode of coordination of the acetic acid, however, also seems possible. (Formula VI). In this formula the two pairs of carbon atoms marked with asterisks

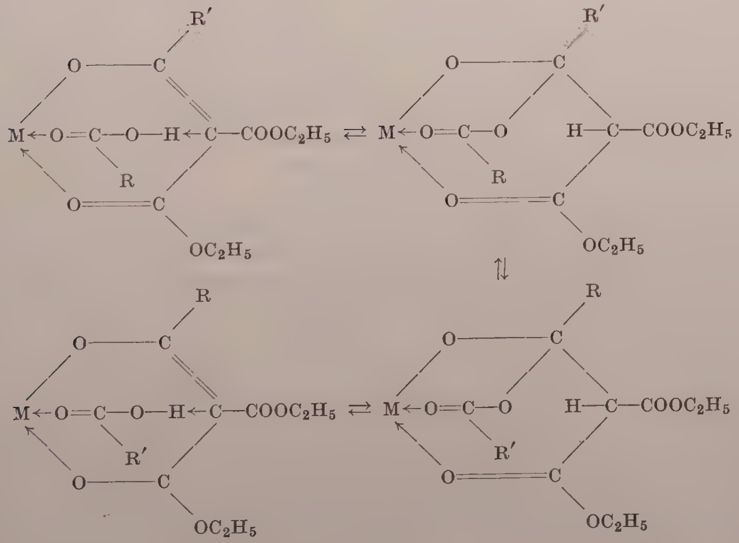


III



$$CH_3-\underset{\underset{O}{\|}}{C}-O-\underset{\underset{O}{\|}}{C}-O-C_2H_5 \longrightarrow CH_3-\underset{\underset{O}{\|}}{C}-O-C_2H_5+CO_2$$

IV



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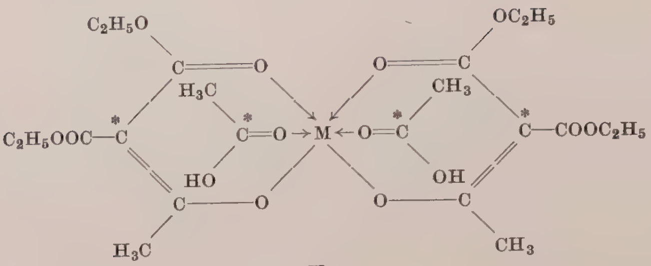
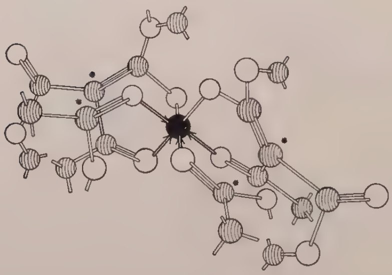


VI



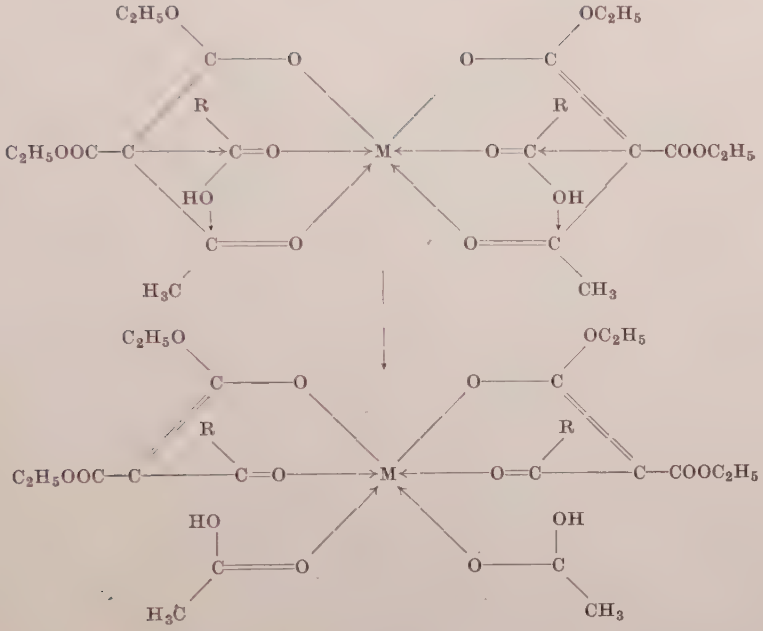
VI a

In this and the following formulae the sterical relations of the groups in the starting materials are based on a study of atomic models, but for the sake of perspicuity this is usually not the case in the reaction products.

will be close to each other, those in the acetic acid with acceptor activity and the others with donor activity. A coordinative link between them appears very probable. Against this view can be argued that the central carbon atom will hold four strongly electronegative groups, which is not usually possible, but it must be remembered that in this case four covalent bonds are never formed and that the coordinative link merely represents a mode of electronic displacement leading to the preferential stabilisation of one of the possible double bond structures in a reactive complex. This stabilisation of a double bond structure will probably effect the sterical distribution of the atoms in the six membered rings so that they are no longer in one plane, as in the resonating structure, but are situated in a manner determinated by the actual double bond structure. This makes it possible to illustrate the sterical relation with the aid of atomic models. (Formulae VI a — X a).

With this formulation, the acyl group exchange (3) can be explained in a very simple way by means of an α, γ-shift of the hydroxyl ion to one of the acyl groups. (Formula VII.) The formation of ethyl diacylacetate (from which a diketone may result by acidolysis) from diethyl acylmalonate can be explained in a similar manner. (Formula VIII.)

It must, however, be pointed out that this mode of formulation does not seem to be able to explain the formation of diethyl malonate from the acidolysis of diethyl acylmalonates (4) or the elimination of a carbethoxy group without replacing it by an acyl group, e.g. the formation of acetylacetone from ethyl diacetoacetate. This is, however, by no means a reason to reject the possibility of at least an acyl exchange by this mechanism; in fact, such a mechanism seems rather probable to the author.



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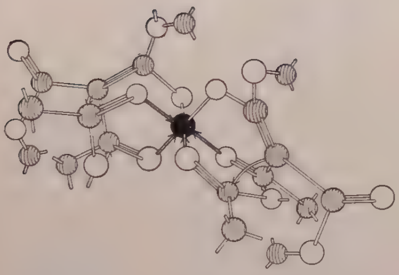
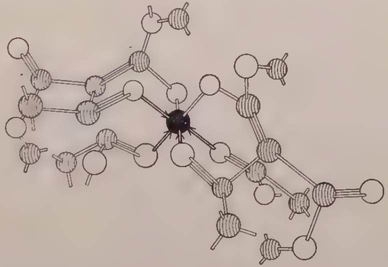


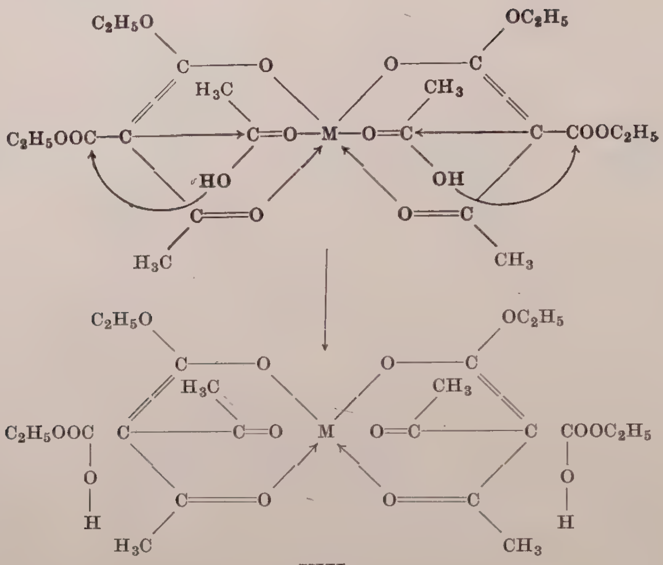
VII a



VII a

The facts given above seem to be in good agreement with the mechanisms suggested, but it must be remembered that most anionoid substitution mechanisms might give the same reaction products, and no conclusive evidence has been given that a coordination of the acid with the central metal atom is necessary for the reactions to proceed.

The fact that the divalent ions of beryllium and copper with the stable maximum coordination number 4 do not catalyse the reaction seems, however, to indicate that such a coordination of the acid is responsible for the reaction, for in these cases no coordination of the acid can take place. The same explanation holds for the trivalent aluminium ion with the coordination number 6. With zinc and cadmium ions the situation is somewhat more complex. These ions might hold six groups by coordination, but they also form compounds in which the coordination number is 4, and in so doing they assume a very stable tetravalent configuration. With this



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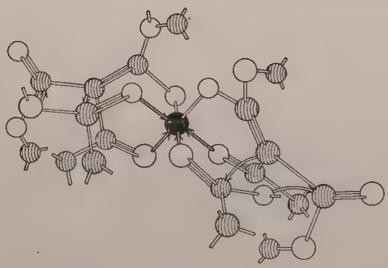

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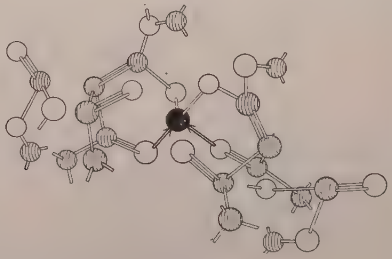
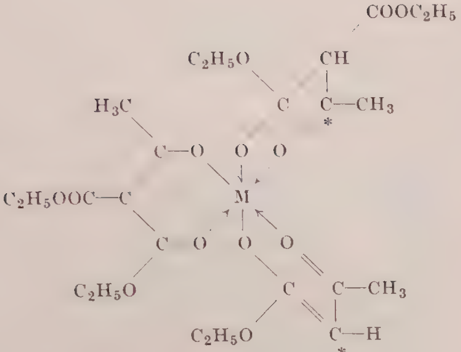


VIII a

structure they will have less tendency than the magnesium ion to coordinate more groups. This is in agreement with the kinetic results. The catalytic effect of the univalent lithium ion and the trivalent boron ion, both with the coordination number 4, is in good agreement with the hypothesis given above.

A very interesting piece of evidence for the coordination mechanism can be obtained from the reaction of diethyl acetylmalonate with ethyl acetoacetate. In this case the coordination number 6 for the magnesium ion may be attained by coordination of one mole of ethyl acetoacetate. In this way the compound IX may be obtained. (Compare with formula VI.) In this molecule, in which the three didentate groups can span only cis positions, the two carbon atoms marked with asterisks will be close to each other. (Formula IX a.) It seems very probable that the same compound might result from the coordination of ethyl acetoacetate to the magnesium chelate of diethyl acetylmalonate, and from the coordination of diethyl acetylmalonate to the mixed magnesium chelate of one mole of ethyl acetoacetate and one mole of diethyl acetylmalonate. If this view is correct the actual state of the molecule will be represented by an equilibrium between the two alter-



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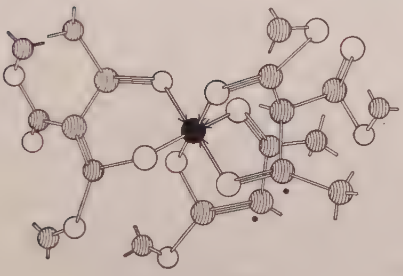
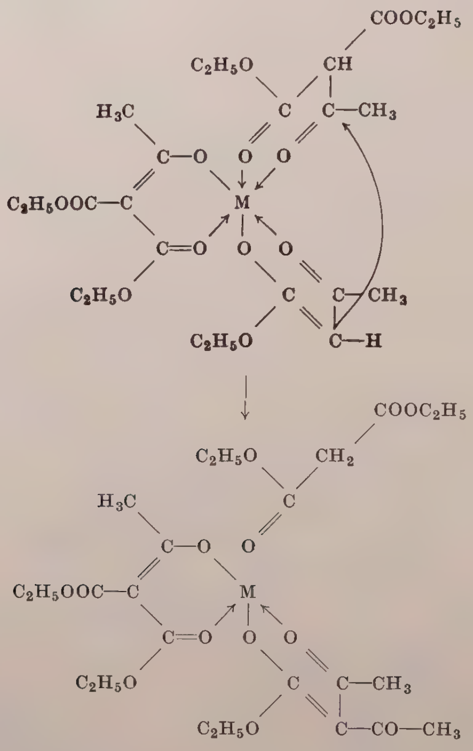


IX a

native modes of formulation. If the latter mode of formulation is considered it can be seen that of the two carbon atoms marked with asterisks the one in the ethyl acetoacetate ring will be a centre of donor activity, while that in the diethyl acetylmalonate ring will be a centre of accepter activity. This makes a formation of a coordinative bond probable between the two opposed activity centers. (Formula X.) From this it is also evident that a change into ethyl diacetoacetate and diethyl malonate can be effected simply by an electronic displacement, followed by a redistribution of the acidic activity to the ethyl diacetoacetate formed.

Experiments with atomic models show that the structure indicated for this complex will be very sensitive to steric effects. The exchange of the methyl groups with *iso*propyl groups or tert-butyl groups will make the formation of such a complex much more difficult. The formation of diethyl malonate and a β-diketone in the synthesis of a β-ketoester from diethyl acylmalonate and an organic acid using magnesium oxide as a catalyst (4) is probably due to the reaction mentioned above. The β-diketone would be a result of the acidolysis of the ethyl diacylacetate formed in this reaction. It has been shown (4) that the ethyl propionylacetate and the ethyl *n*-butyrylacetate obtained from the corresponding diethyl acylmalonates are contaminated with considerable quantities of the β-diketones and diethyl malonate, but the ethyl *iso*butyrylacetate so obtained is almost entirely free from these products. A synthesis of ethyl pivaloylacetate by the same method shows that this product is obtained free from diethyl malonate and dipivaloylmethane. This is in good agreement with the mechanism proposed.



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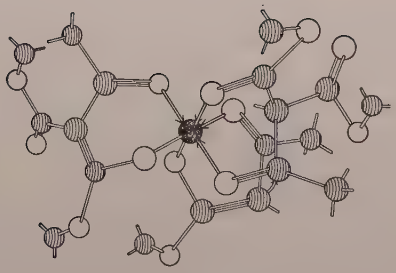

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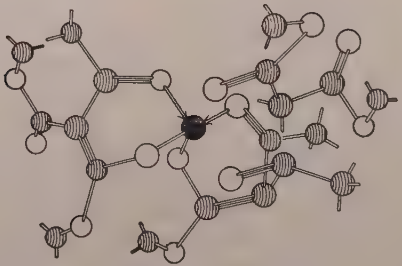

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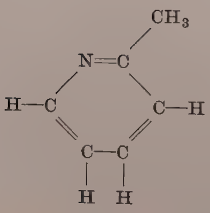

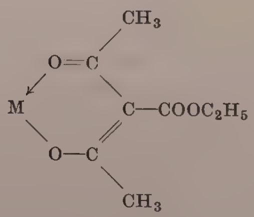

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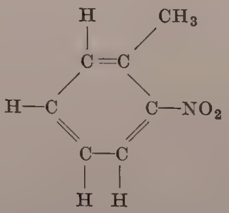

Table 1.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
60	97	0.0134	0.6533	0.3199	0.3101
120	200	0.0277	0.6390	0.3056	0.3203
180	297	0.0411	0.6256	0.2922	0.3306
255	415	0.0574	0.6093	0.2759	0.3440
305	488	0.0675	0.5992	0.2658	0.3530
365	571	0.0790	0.5877	0.2543	0.3638
420	642	0.0888	0.5779	0.2445	0.3736
480	708	0.0979	0.5688	0.2354	0.3832
541	778	0.1076	0.5591	0.2257	0.3940
600	844	0.1167	0.5500	0.2166	0.4047

2.40 mg MgO $V_\infty = 2410$ ml $K = 1.21 \cdot 10^{-3}$

Table 2.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	97	0.0136	0.6531	0.3197	0.3103
60	196	0.0274	0.6397	0.3059	0.3204
90	295	0.0412	0.6255	0.2921	0.3307
120	386	0.0540	0.6127	0.2793	0.3412
150	474	0.0663	0.6004	0.2670	0.3519
180	557	0.0779	0.5888	0.2554	0.3628
240	711	0.0994	0.5667	0.2339	0.3844
270	778	0.1088	0.5579	0.2245	0.3954
300	843	0.1179	0.5488	0.2154	0.4061
330	909	0.1271	0.5396	0.2062	0.4178
360	966	0.1351	0.5316	0.1982	0.4285

8.97 mg MgO $V_\infty = 2384$ ml $K = 2.50 \cdot 10^{-3}$

Table 3.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	151	0.0204	0.6463	0.3129	0.3150
60	299	0.0404	0.6263	0.2929	0.3301
90	443	0.0598	0.6069	0.2735	0.3461
120	574	0.0775	0.5892	0.2558	0.3624
150	693	0.0936	0.5731	0.2397	0.3785
180	804	0.1085	0.5582	0.2248	0.3950
210	905	0.1222	0.5445	0.2111	0.4115
240	997	0.1346	0.5321	0.1987	0.4278

19.75 mg MgO $V_\infty = 2469$ ml $K = 3.75 \cdot 10^{-3}$

Table 4.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
10	78	0.0103	0.6564	0.3230	0.3080
20	154	0.0204	0.6463	0.3129	0.3150
30	232	0.0307	0.6360	0.3026	0.3226
40	305	0.0404	0.6263	0.2929	0.3301
50	381	0.0505	0.6162	0.2828	0.3382
60	456	0.0604	0.6063	0.2729	0.3467
70	527	0.0698	0.5969	0.2635	0.3551
80	592	0.0785	0.5882	0.2548	0.3633
90	658	0.0872	0.5795	0.2461	0.3720
100	720	0.0954	0.5713	0.2379	0.3805
110	777	0.1030	0.5637	0.2303	0.3888
120	834	0.1105	0.5562	0.2228	0.3973
130	889	0.1178	0.5489	0.2155	0.4060
140	940	0.1246	0.5421	0.2087	0.4146
150	983	0.1303	0.5364	0.2030	0.4220
44.13 mg MgO $V_\infty = 2515$ ml $K = 5.82 \cdot 10^{-3}$					

Table 5.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
10	101	0.0137	0.6530	0.3196	0.3103
20	198	0.0269	0.6398	0.3064	0.3197
30	290	0.0394	0.6273	0.2939	0.3293
40	381	0.0518	0.6149	0.2815	0.3393
50	470	0.0639	0.6028	0.2694	0.3498
60	551	0.0749	0.5918	0.2584	0.3599
70	631	0.0858	0.5809	0.2475	0.3705
80	703	0.0956	0.5712	0.2377	0.3808
90	773	0.1051	0.5616	0.2282	0.3911
100	839	0.1141	0.5526	0.2192	0.4016
110	903	0.1228	0.5439	0.2105	0.4122
120	958	0.1303	0.5364	0.2030	0.4220
71.04 mg MgO $V_\infty = 2451$ ml $K = 7.14 \cdot 10^{-3}$					

Table 6.

mg MgO	2.40	8.97	19.75	44.13	71.04
m.mol. MgO	0.0595	0.2225	0.4899	1.094	1.762
(m.mol. MgO)$^{1/2}$ ..	0.244	0.472	0.699	1.046	1.327
$K \cdot 10^{+3}$	1.21	2.50	3.75	5.82	7.14

Table 7.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
120	69	0.0095	0.3238	$\bar{1}.5103$
240	140	0.0194	0.3139	$\bar{1}.4968$
365	218	0.0301	0.3032	$\bar{1}.4817$
420	252	0.0348	0.2985	$\bar{1}.4749$
600	352	0.0487	0.2846	$\bar{1}.4542$
1380	742	0.1024	0.2309	$\bar{1}.3634$
1440	771	0.1066	0.2267	$\bar{1}.3555$
1575	828	0.1145	0.2188	$\bar{1}.3401$
1680	873	0.1207	0.2126	$\bar{1}.3276$
1815	927	0.1282	0.2051	$\bar{1}.3120$

21.5 mg Li_2CO_3 $V_\infty = 2411$ ml $K = 2.695 \cdot 10^{-4}$

Table 8.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
60	73	0.0100	0.3233	$\bar{1}.5096$
120	145	0.0199	0.3134	$\bar{1}.4961$
180	221	0.0304	0.3029	$\bar{1}.4813$
240	295	0.0406	0.2927	$\bar{1}.4664$
360	434	0.0597	0.2736	$\bar{1}.4371$
420	498	0.0685	0.2648	$\bar{1}.4229$
480	564	0.0776	0.2557	$\bar{1}.4077$
540	624	0.0858	0.2475	$\bar{1}.3936$
600	687	0.0945	0.2388	$\bar{1}.3780$
660	741	0.1019	0.2314	$\bar{1}.3644$

60.4 mg Li_2CO_3 $V_\infty = 2423$ ml $K = 5.610 \cdot 10^{-4}$

Table 9.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
60	127	0.0174	0.3159	$\bar{1}.4996$
120	235	0.0323	0.3010	$\bar{1}.4768$
180	347	0.0477	0.2856	$\bar{1}.4558$
240	454	0.0624	0.2709	$\bar{1}.4328$
300	555	0.0763	0.2570	$\bar{1}.4099$
360	650	0.0893	0.2440	$\bar{1}.3874$
420	745	0.1024	0.2309	$\bar{1}.3634$
480	828	0.1138	0.2195	$\bar{1}.3414$
540	913	0.1254	0.2079	$\bar{1}.3179$

110.7 mg Li_2CO_3 $V_\infty = 2426$ ml $K = 8.74 \cdot 10^{4}$

Table 10.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
60	147	0.0198	0.3135	$\bar{1}.4962$
120	282	0.0380	0.2953	$\bar{1}.4703$
180	415	0.0560	0.2773	$\bar{1}.4430$
240	541	0.0730	0.2603	$\bar{1}.4156$
300	663	0.0894	0.2439	$\bar{1}.3872$
360	778	0.1049	0.2284	$\bar{1}.3587$
420	882	0.1189	0.2144	$\bar{1}.3312$
142.7 mg Li_2CO_3 $V_\infty = 2472$ ml $K = 10.55 \cdot 10^{-4}$				

Table 11.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	97	0.0133	0.3200	$\bar{1}.5052$
65	184	0.0252	0.3081	$\bar{1}.4887$
90	245	0.0336	0.2997	$\bar{1}.4767$
120	321	0.0440	0.2893	$\bar{1}.4614$
150	398	0.0546	0.2787	$\bar{1}.4451$
180	472	0.0647	0.2686	$\bar{1}.4291$
210	548	0.0751	0.2582	$\bar{1}.4120$
240	620	0.0850	0.2483	$\bar{1}.3950$
270	688	0.0943	0.2390	$\bar{1}.3784$
300	754	0.1034	0.2299	$\bar{1}.3615$
330	818	0.1122	0.2211	$\bar{1}.3446$
390	944	0.1294	0.2039	$\bar{1}.3094$
192.4 mg Li_2CO_3 $V_\infty = 2431$ ml $K = 12.61 \cdot 10^{-4}$				

Table 12.

mg Li_2CO_3	21.5	60.4	110.7	142.7	192.4
m.mol. Li_2CO_3 ...	0.291	0.817	1.498	1.931	2.604
(m.mol. $Li_2CO_3)^{3/4}$	0.3962	0.8597	1.354	1.638	2.003
$K \cdot 10^4$	2.695	5.610	8.74	10.55	12.61

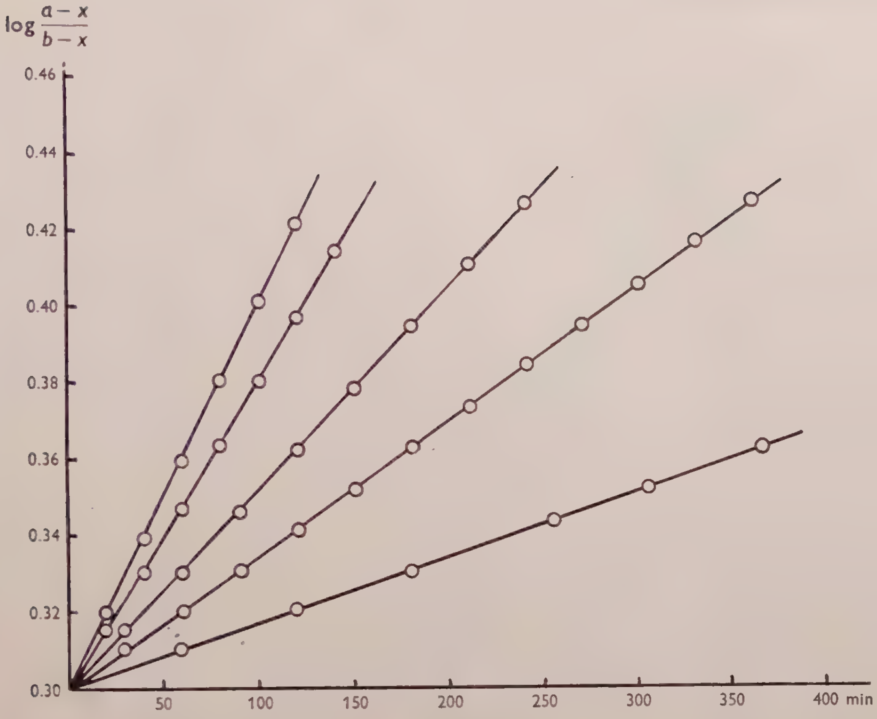

Fig. 1.

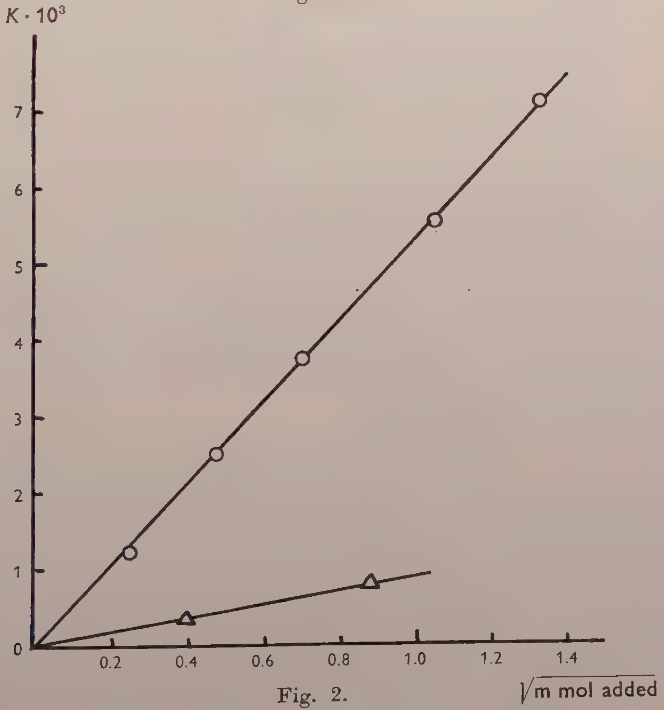

Fig. 2.

○ = Mg O added, △ = Zn $(CH_3\ COO)_2$ added.

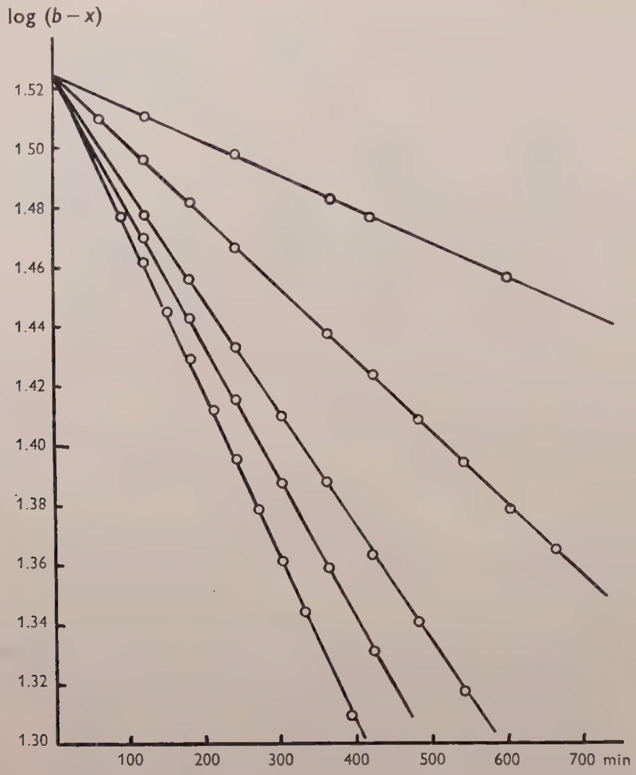

Fig. 3.

The theoretical explanation given above does not consider the activation of the alkyl groups in the side chains of the chelate rings. It is very probable that such an activation will exist which will be seen by a comparation of the formulae XI. In both comparing substances the methyl group is highly activated. Experiments on the reaction of an ester with diethyl acetylmalonate indicated a certain reactivity of the methyl group in this compound. This reaction will be the subject of a future investigation, but for the present purposes it can be mentioned that the reaction seems to be rather slow and will probably be of secondary importance in comparison with the other rather rapid reactions.

From the experimental material available (1–4) and the theoretical explanations given above it can be predicted that the acidolysis of diethyl acylmalonates using magnesium oxide as a catalyst will give good yields of branched chained β-ketoesters of a high purity. In order to minimize the extent of the formation of diethyl malonate in the syntheses of the more simple β-ketoesters it seems favorable to use an excess of the acid in the reaction. This would depress the formation of the coordination compounds containing the β-ketoesters primarily formed.

The acyl exchange observed in the reaction of ethyl diacetoacetate with *n*-butyric acid indicates that the syntheses of unsymmetrical β-diketones $RCOCH_2COR'$ by the magnesium oxide catalysed acidolysis of ethyl diacylacetates (5) must be carried

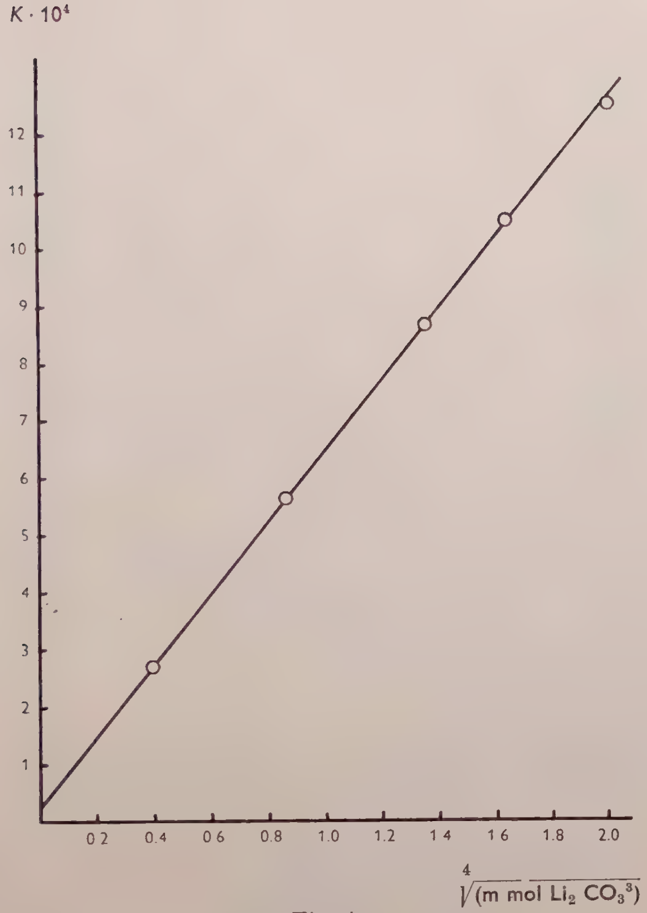

Fig. 4.

out with considerable skill in order not to get impure products. In the syntheses of symmetrical β-diketones $RCOCH_2COR$ the method using diethyl malonate and an acid anhydride (2) will be very convenient and give products of a good purity.

Experimental

The kinetic studies were performed at 120.0° using 0.1 mole of ethyl diacetoacetate, 0.2 moles of glacial acetic acid and varying amounts of the catalysts. The ester and the acid were mixed in a 100 ml flask, fitted with an efficient reflux condenser and connected to the top of a 1000 ml gas buret using water saturated with carbon dioxide as a barring liquid. The mixture was heated for 2 hours in the thermostat and a very small beaker containing the catalyst was then added. The progress of the reaction was then followed by measuring the volume of carbon dioxide formed.

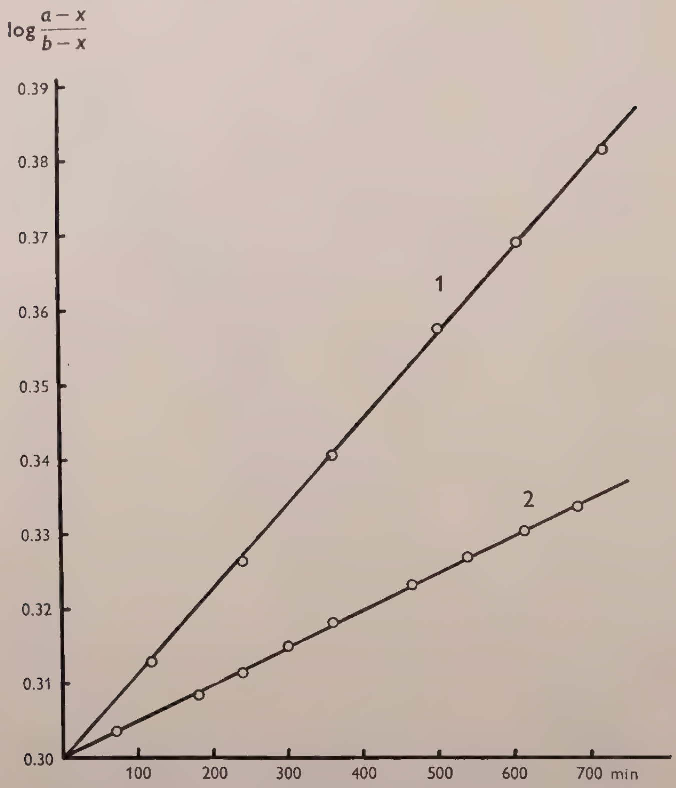

Fig. 5.

1. 0.775 m mol Zn $(CH_3COO)_2$; $K=7.8\cdot10^{-4}$
2. 0.153 m mol Zn $(CH_3COO)_2$; $K=3.4\cdot10^{-4}$.

In order to obtain the moles of carbon dioxide formed the limiting volume V_∞ was calculated in the usual way. (Standard molar volume of carbon dioxide 22.264 l.)

In the tables x, $a-x$, and $b-x$ are the mole fractions of acetylacetone or carbon dioxide, acetic acid and ethyl diacetoacetate respectively.

The results obtained with magnesium oxide and lithium carbonate are given in tables 1–12 and figures 1–4.

The results obtained with zinc acetate are given in fig. 5 and compared with these with magnesium oxide in fig. 2.

With boric acid as the catalyst the reaction product became very deeply coloured and the measurements did not give good results. The velocity variations with the concentrations of acetic acid and that of ethyl diacetoacetate could not be determinated with any certainty. The reaction velocity seems, however, to be inversely proportional to the second power of the amount of acetylacetone formed in the reaction. For such a reaction the following equations are valid:

$$\frac{dx}{dt}=\frac{K}{x^2} \text{ or } x=\sqrt[3]{3\,Kt}.$$

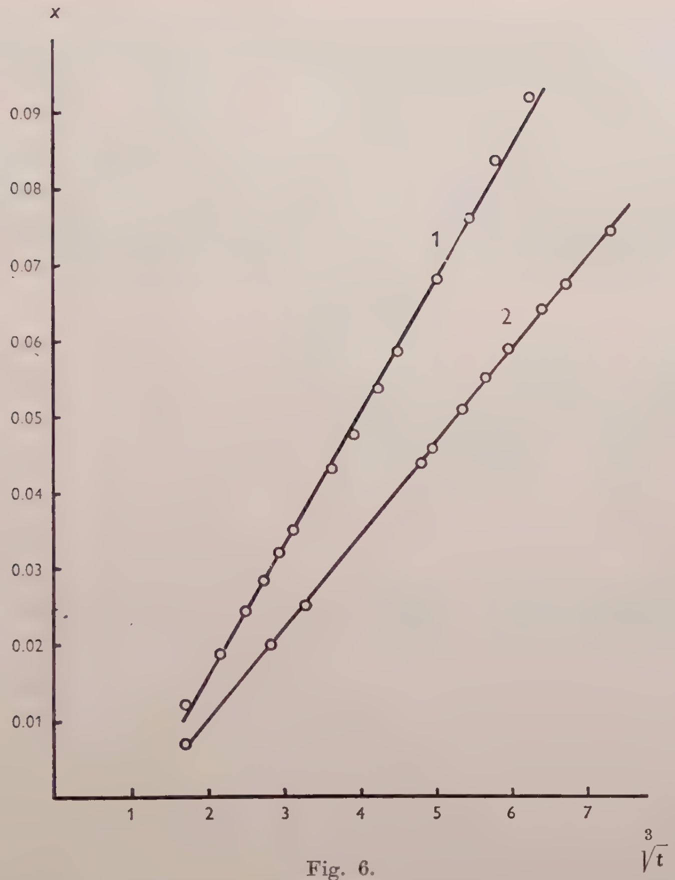

Fig. 6.

1. 428.5 mg H_3BO_3 added

2. 229.2 mg H_3BO_3 added.

According to this the plot of x against $\sqrt[3]{t}$ should give a straight line. This is shown in fig. 6 for two additions of boric acid.

The retarding effect of acetylacetone may perhaps be explained as a preferential coordination of acetylacetone to the boron atom.

The kinetic method used seems to give such good results with magnesium oxide and lithium carbonate as to merit its further use in this reaction.

Preparations

Ethyl pivaloylacetate was prepared from 68 g of diethyl pivaloylmalonate b.p. 139°/10 mm and 32 g of pivalic acid according to the general method (1). In this way 32 g of ethyl pivaloyl acetate was obtained (67% yield). The boiling point of the product was 87–87.5°/8 mm, indicating a very pure product, and even with the aid of a very efficient column no forefraction containing diethyl malonate could be obtained.

The acyl exchange between ethyl diacetoacetate and butyric acid was studied using 0.2 moles of each component. The mixture was refluxed with 0.2 g of magnesium oxide as a catalyst. When the vigorous evolution of carbon dioxide had subsided the mixture was distilled through a very efficient column. In this way, a 33% yield of ethyl acetate b.p. 76–77° was obtained together with 17 g of a fraction boiling at 95–120° mostly at 118.5° and consisting mainly of ethyl *n*-butyrate together with some acetylacetone. From the residue the β-diketones were precipitated as copper derivatives from which the expected β-diketones acetylacetone b.p. 139° and *n*-butyrylacetone b.p. 174° could be liberated, in the usual way.

Summary

The acidolysis of ethyl diacetoacetate and diethyl acetylmalonate has been studied. A reaction mechanism which would explain the great variety of products obtained in this reaction has been suggested. The first step in the reaction has been assumed to be the formation of a complex compound in which the reacting groups are in suitable positions to each other. The reaction will then take place merely by an electronic displacement or a α, γ-shift of a simple group.

The author is indebted to Mr Ragnar Öhberg for preparing the ethyl diacetoacetate, and to Mrs Margareta Englund and Mr Nils-Tage Nordin for valuable help with the illustrations.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, May 1952.

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Isotope effect of hydrogen and mechanism of aromatic sulphonation

By Ulla Berglund-Larsson and Lars Melander

With 2 figures in the text

In an earlier paper (1) evidence was obtained of an isotope effect of hydrogen in aromatic sulphonation, while nitration was found to proceed with the same velocity irrespective of the mass of the expelled hydrogen. In the case of sulphonation the accuracy of the measurements was rather low owing to the fact that the deviations were close to the limits of experimental error. Moreover, another point of uncertainty was introduced by the low solubility of the aromatic compound in the sulphuric acid and consequently the lack of homogeneity under certain conditions. The purpose of the present investigation was to get a reliable determination of the existence and magnitude of the isotope effect for the couple tritium-protium.

When tracer amounts of hydrogen are used as in the case of tritium, it is desirable that the substitution should take place in as few competing equivalent positions as possible, especially in the case of sulphonation, in which the yield often is far from quantitative. The reasons have been given previously (1a). Sulphonation of a benzene derivative containing an ortho-para-directing group will in some cases give almost exclusively the para derivative. This is the case with bromobenzene, which was chosen for the present experiments in spite of its low solubility in oleum. Nitrobenzene with its two meta positions available for sulphonation would have been more favourable as to the solubility, but the difficulty of preparing nitrobenzene-3-t prevented its use. The latter difficulty applies also to 1,3-dinitrobenzene-5-t.

Sulphonation in oleum as solvent

The first series of experiments was mainly carried out in the same manner as in the previous investigation referred to above (1b), i.e. with the sulphonating agent itself as the solvent.

When the isotopes of hydrogen are replaced with different rates by a substituent, i.e. when an isotope effect exists, the ratio between the isotopic molecules in the reactants will vary continuously during the course of the reaction. For that reason it is necessary to know both the isotopic proportion and how far the reaction has proceeded for a calculation of the isotope effect.

The reaction was quenched by pouring out into ice water. A known amount of the separated remaining bromobenzene was pipetted off for the determination of its specific tritium content, and a known amount of ordinary, inactive bromobenzene was added and the mixture shaken vigorously. The specific activity of the final bromobenzene together with that of the first sample and the known volumes gave the amount of bromobenzene which had not reacted. If the formation of by-products may be neglected (which is a reasonable assumption under these conditions), the yield of sulphonic acid is obtained from this amount.

The reaction in oleum was rather rapid, and owing to the evolution of heat it was difficult to control the temperature. It seems likely that the irregularities in the results (cf. Discussion below) were caused by the separation of the reaction mixture into two phases. As it was difficult to find conditions under which the rate of reaction was convenient and at the same time the compounds did not separate, oleum was abandoned as a solvent.

Sulphonation with oleum in nitrobenzene

Experiments with sulphur trioxide and fuming sulphuric acid as sulphonating agents in different solvents, e.g. chloroform, dioxane, carbon disulphide, and nitrobenzene were carried out. Only nitrobenzene, previously used by HINSHELWOOD *et al.* in several investigations, fulfilled the requirements that the solvent should not be attacked by the sulphonating agent and that the reaction rate should be moderate at suitable concentrations and temperatures. The following experiments were carried out with rather dilute solutions in nitrobenzene of fuming sulphuric acid with up to 21 per cent anhydride, and the amount of oleum was such that anhydride was still present when the reaction was stopped. The temperature was kept at 0° C. The course of the reaction was followed by means of alkalimetric titration.

When the reaction was to be stopped, the mixture was poured out into several times the volume of cold water and stirred. The mixture was made alkaline. The remaining bromobenzene was separated from the nitrobenzene phase by means of distillation, and in some cases the sulphonate was obtained from the aqueous phase by means of evaporation and recrystallization.

Control experiments. It is a necessary condition for the measurement of the isotope effect that no spontaneous hydrogen exchange takes place during the reaction. As is well known (2), hydrogen exchange is more rapid than sulphonation in aqueous sulphuric acid of intermediate concentration. In strong oleum, however, the opposite holds (1 c). In order to investigate the exchange in nitrobenzene solution two sulphonations with oleum containing tritium were carried out on inactive bromobenzene under the ordinary conditions and with rather high yields. The remaining bromobenzene was practically free of tritium. In one of the experiments the nitrobenzene was examined for tritium and was found to contain none. The experiments prove simultaneously that the stopping of the sulphonation cannot be due to an appreciable reverse reaction, for in such a case the regenerated bromobenzene should have contained tritium in fairly large amounts.

In two experiments 100 per cent sulphuric acid was used in nitrobenzene solution. In order to give a convenient reaction velocity these mixtures were kept at 80° C. When bromobenzene-4-t was used, tritium occurred in the resulting p-bromobenzenesulphonate. This is not the case under ordinary conditions. Thus the tritium must have changed its place. When inactive bromobenzene was used with tritium in the sulphuric acid, tritium was found in the remaining bromobenzene after only 13 per cent reaction in an amount not far from equilibrium. Under these conditions hydrogen exchange seems to be faster than sulphonation, and the bromobenzene-t was hardly formed in a reversal of the latter.

When the reaction is quenched in water, the sulphuric acid is momentarily diluted. In order to show that no exchange occurs in the dilution moment, a sulphonation mixture with bromobenzene-4-t was quenched at zero time. The bromobenzene was recovered with its specific acitvity unchanged.

One experiment was carried out in order to determine the amount of reaction with the solvent under ordinary conditions. During a period corresponding to more than 12 times the maximum reaction time used in the ordinary experiments, the decrease in acid titre was within the titration error.

Experimental

Tritium. The tritium used in the present investigation was obtained partly from the small cyclotron of the Nobel Institute of Physics, Stockholm (3), and partly from the Atomic Energy Research Establishment, Harwell.

Measurement of tritium. The measurements of tritium were carried out with the same apparatus as used in earlier investigations (3, 1 d). All organic compounds were burned and the water reduced to hydrogen, which was used for inside counting in a Geiger-Müller tube. The accuracy of the determinations is within ± 2 %. The unit of specific activity used throughout the investigation is referred to the Geiger-Müller tube used and corresponds to about 250 disintegrations per millimole of hydrogen per minute. The corrections for the natural decay of tritium were made assuming its half-life to be 12.5 years.

The organic preparations were burned in oxygen over either platinized asbestos, silver wool and Vinosit B in one tube or red hot cupric oxide with a second tube containing heated silver wool and a second layer of cupric oxide. When 2,4-dinitro bromobenzene was burned, the latter method was used, a third tube with heated copper being intercalated, and the oxygen atmosphere replaced by carbon dioxide. The solid sodium sulphonate was mixed with molten and powdered potassium dichromate in a copper boat before burning.

Preparation of bromobenzene-4-t. Bromobenzene-4-t was prepared from *p*-dibromobenzene by means of decomposition of the corresponding Grignard compound with an ether solution of $HCl + H_2O$ containing tritium (1 e). The addition of benzene is helpful in the Grignard reaction.

For a control of the isomeric purity of the two preparations of bromobenzene-4-t used in nitrobenzene solution samples were converted into 2,4-dinitrobromobenzene (1 f). The preparations contained less than 1.7 and 0.8 % of the tritium in positions other than para. The values are calculated on the assumption of contamination by the ortho isomer. If meta is assumed the values should be halved. The purification of the *p*-dibromobenzene seems to be decisive.

The radioactive bromobenzene preparations were diluted with a suitable amount of inactive compound (Grave, chem. pure, S.F., for the preparation used in oleum as solvent; Merck & Co., U.S.A., anal. reagent, for the preparation used in nitrobenzene).

Fuming sulphuric acid. Different commercial fuming sulphuric acids were used (Kebo, p. a., 10–12 % SO_3; Riedel de Haën, p. a., 10 % SO_3; Baker analyzed, c:a

15 % SO_3 and 20–23 % SO_3). For the determination of free SO_3, known proportions of water were added, and from the freezing point curve obtained the amount of water equivalent to the free SO_3 was determined (pure H_2SO_4 corresponding to maximum freezing point) (4).

Sulphonation in oleum as solvent.[1] 2.5 or 3 ml of bromobenzene-4-t was placed in a small separate container in a flask with a ground stopper. 3 to 6 ml of fuming sulphuric acid (10–12 % free SO_3) was introduced in the other compartment and the flask stoppered. It was cooled with water before mixing and then shaken with water cooling for about a minute, after which the shaking was continued for different intervals at ordinary temperature. The mixture was poured out into ice water and the residue washed out of the flask. From the bromobenzene phase 0.5 ml was pipetted off for tritium determination and 1 or 2 ml of inactive bromobenzene added with a pipette to the remaining mixture. The mixture was shaken thoroughly and finally a new sample of bromobenzene taken.

The bromobenzene samples were washed with sodium carbonate solution and water, dried with calcium chloride, and finally distilled under reduced pressure. The index of refraction was checked.

Sulphonation in nitrobenzene. Weighed amounts of nitrobenzene (250 ml) and oleum (15 ml) were introduced into a flask and the concentration of acid controlled by means of titration (see below). After the titration the mixture was weighed again and cooled to 0° C. 5.0 ml of bromobenzene-4-t of 0° C was added, the mixture being placed in an ice-bath. The solution was magnetically stirred during the reaction. Samples were withdrawn now and then to follow the reaction.

The samples (about 1 ml) were poured out into a weighed mixture of 50 ml of water and 30 ml of acetone, the mixture weighed again, and more acetone added till the solution became perfectly clear. The solution was titrated with 0.1 N sodium hydroxide with methyl red or neutral red as indicator, the latter giving the sharpest colour change under these conditions.

In order to eliminate the titration errors as far as possible several titrations were made during the course of the reaction and a smooth curve was drawn representing the progress of the reaction as a function of time. The intersection of the curve with the time at which the reaction was stopped was taken as the yield.

The reaction was quenched in 3 to 4 volumes of cold water, the mixture stirred vigorously and made alkaline with sodium hydroxide. The phases were separated.

The nitrobenzene phase was further washed with sodium carbonate solution and water and dried with calcium chloride. The bromobenzene was distilled off under reduced pressure in an empty vacuum-jacketed Widmer column. It was distilled twice more under reduced pressure in an ordinary Claisen flask. The boiling point and the index of refraction of the final preparation agreed well with that of pure bromobenzene.

When the sulphonate was to be investigated, the filtered aqueous phase was made just neutral with sulphuric acid and evaporated to dryness. The residue was powdered and extracted three times with boiling absolute ethanol. The extract was filtered and evaporated to dryness. The residue was recrystallized from the same solvent and dried over phosphorus pentoxide under reduced pressure. The product seemed to be free of sulphone, as extraction with warm ether gave no residue on evaporation of the extract.

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[1] We take this opportunity to thank Ing. KJELL HALVARSON for carrying out the main part of these experiments.

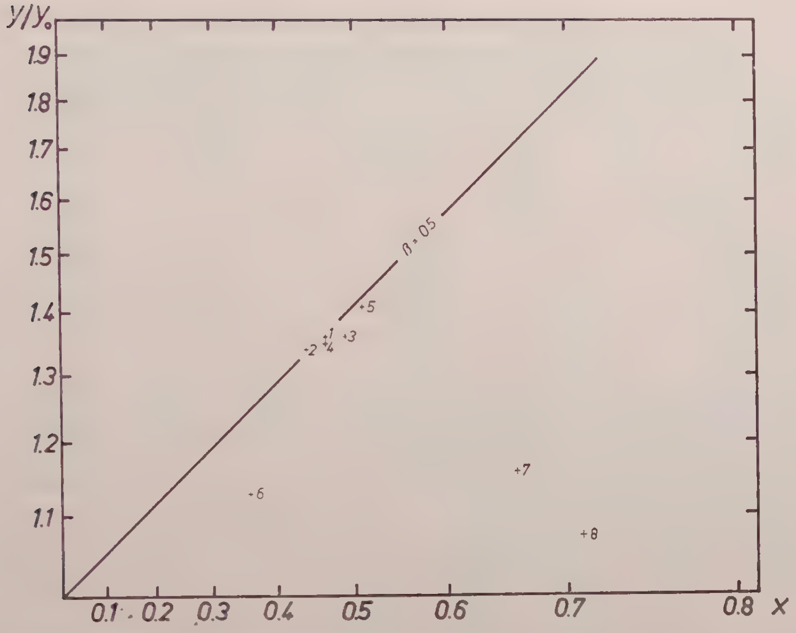


Fig. 1. Double-logarithmic representation of the relative change in specific activity (y/y_0) of remaining bromobenzene-4-t as a function of the fraction sulphonated (x) in oleum as solvent. Cf. Table I.

The conditions of some control experiments are given together with the results in the next section.

Results

The relative change in the specific activity of the bromobenzene during the reaction is represented by the equation

$$\frac{y}{y_0} = (1-x)^{-(1-\beta)}$$

where y_0 denotes the specific activity of the original bromobenzene and y that corresponding to the moment when the fraction x of the bromobenzene has reacted. β is the ratio k_T/k_H, k_T and k_H being the specific rates of reaction of the tritium compound and the ordinary one (1 g). The results obtained may conveniently be represented in a double-logarithmic diagram:

$$\log \frac{y}{y_0} = -(1-\beta) \log (1-x).$$

If the isotope effect is independent of the yield, as it should, points corresponding to different yields should be situated on the same straight line through the origin. β is obtained from its slope.

Table I. Data of sulphonations in oleum as solvent, cf. Fig. 1.

Exp.	Oleum, 10–12% free SO_3, ml	Bromobenzene ml	Time of reaction, min
1	3	3	2
2	3	3	3
3	3	3	4
4	3	3	5
5	3	3	6
6	5	2.5	0.5
7	6	3	20
8	6	3	40

The results from the sulphonation in oleum as solvent are represented in Fig. 1 and the conditions of the corresponding individual experiments are found in Table 1. The two coordinates in the diagram are not completely independent of one another experimentally but have one specific activity determination in common. It might be shown that the significant deviations fall well beyond the experimental errors.

The results from the sulphonations in nitrobenzene are given in Fig. 2 and the corresponding conditions in Table II. The limits of error given in Fig. 2 are those of the titration and the tritium determination, respectively.

Control experiments. In two ordinary sulphonations 15 mg of water containing tritium was added. The data are given in Table III.

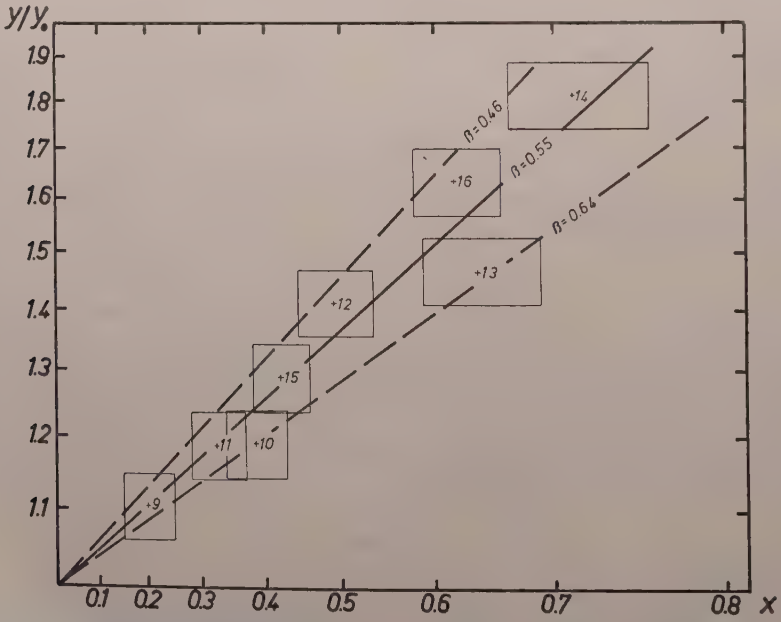


Fig. 2. Double-logarithmic representation of the relative change in specific activity (y/y_0) of remaining bromobenzene-4-t as a function of the fraction sulphonated (x) in nitrobenzene solution. Cf. Table II.

Table II. Data of sulphonations in nitrobenzene, cf. Fig. 2.

For amounts of reactants, see Experimental. Temp. 0° C.

Exp.	Mole fraction of free SO_3 in total mixture		Mole fraction of H_2SO_4 in total mixture*	Time, min	n_D of final bromobenzene
	Initial	Final*			
9	0.0187	0.0155	0.0833	21	1.5585 (23° C)
10	0.0188	0.0119	0.0855	63	1.5573 (24.5° C)
11	0.0163	0.0105	0.0849	95	1.5589 (21° C)
12	0.0183	0.0094	0.0925	96	1.5590 (20° C)
13	0.0191	0.0077	0.0851	193	1.5583 (22° C)
14	0.0183	0.0060	0.0854	440	1.5570 (24.5° C)
15	0.0248	0.0175	0.0817	36	1.5580 (23° C)
16	0.0250	0.0133	0.0815	69	1.5580 (23.5° C)

* The change in total number of molecules as a result of the reaction has been neglected.

Table III. Control against exchange in sulphonation in nitrobenzene.

s = spec.act. averaged over all hydrogens in the system.
s_1 = average spec.act. of bromobenzene hydrogens, the activity first being averaged over oleum and 1 of the initial bromobenzene hydrogens.
s_3 = foregoing but with 3 initial bromobenzene hydrogens.
s_5 = foregoing but with 5 initial bromobenzene hydrogens.
$s_{C_6H_5Br}$ and $s_{C_6H_5NO_2}$ = observed final average spec.act. of hydrogen in resp. molecule.

Exp.	Mole fraction of free SO_3 in total mixture		Mole fraction of H_2SO_4 in total mixture*	Time min	Yield %	Calculated				Observed	
	Initial	Final*				s	s_1	s_3	s_5	$s_{C_6H_5Br}$	$s_{C_6H_5NO_2}$
17	0.0194	0.0096	0.0864	120	57	5.3	26	65	94	0.2	—
18	0.0197	0.0063	0.0870	1200	76	5.2	25	64	92	0.6	<0.05

* Cf. foot-note of Table II.

Appreciable hydrogen exchange was obtained in the two following experiments. A mixture of 100 % sulphuric acid (mole fraction 0.204), bromobenzene (mole fraction 0.092) containing bromobenzene-4-t, and nitrobenzene was kept at 80° C for 22 hours. The yield calculated on bromobenzene was 17.6 %. The initial specific activity of the bromobenzene was 36.5, the final 17.9, and that of the sodium p-bromobenzenesulphonate 8.6, all figures being average specific activities for the total hydrogen of the molecules.

In a similar experiment a mixture of 100 % sulphuric acid (mole fraction 0.179) containing tritium, inactive bromobenzene (mole fraction 0.093), and nitrobenzene was kept at 80° C for 23 hours. The yield calculated on bromobenzene was 12.8 %. If the specific activity is averaged over the initial sulphuric acid and 1, 3 or 5 positions of the initial bromobenzene, the average specific activity of the hydrogens of the latter would have been 48, 101, and 131 respectively. The experimental value was 118.

The control experiment against hydrogen exchange at the instant of dilution was carried out on an ordinary mixture of the same type as in experiments 9–14 (Table II).

The time of reaction was of the order of 2 minutes at 0° C. The initial specific activity of the bromobenzene was 37.6 and the final was found to be 37.7.

The determination of the reaction of the sulphuric acid with the solvent only was carried out in a mixture similar to those of experiments 15–16 (Table II) at 0° C. After 5700 minutes the decrease in acid titre was too small to be determined accurately but was certainly less than 0.16 % of the total titre. This corresponds to a loss of 1.3 % of the free SO_3, calculated on the assumption that the decrease was due to the formation of sulphonic acid.

Discussion

The control experiments against hydrogen exchange during the reaction (Table III) show that both the bromobenzene and the solvent nitrobenzene are practically stable towards exchange during the sulphonation in this solvent with oleum of sufficiently high anhydride concentration. The final bromobenzene of experiments 17 and 18 has a slight tritium content, which might be due to either exchange or reversal of the sulphonation. The absence of tritium from the nitrobenzene in experiment 18 shows that the calculated figures s_1, s_3, and s_5 but not s should be used for comparison. In consideration of this fact it might be stated that the amount of exchange or reversal of the sulphonation is very small. If the reverse reaction were the cause of the slowing down of the forward reaction, the bromobenzene should have contained considerable amounts of tritium, especially in experiment 18, in which the forward reaction had become very slow.

The experiments with originally 100 per cent sulphuric acid were carried out under conditions different from those of the main experiments in order to get a suitable reaction velocity with this weak acid. They show a considerable hydrogen exchange, which cannot be explained by a reversal of the sulphonation reaction alone, for in such a case the sulphonate could not have contained tritium. If there were no exchange with the bromobenzene, it is extremely impropable that there would be any with the sulphonic acid. Even under the ordinary conditions a similar exchange seems to occur at too small concentrations of anhydride, for in some experiments not described here with a mole fraction of anhydride of about half the value in experiments 9–14, values of y/y_0 slightly inferior to 1 were obtained although the free anhydride was never used up in the reaction.

From Fig. 2 a β value of 0.55 ± 0.09 at 0° C is obtained. There is no obvious trend caused by differences in the fraction of bromobenzene converted or by differences in anhydride concentration.

The points in Fig. 1 are not situated on a straight line even if the experimental errors in the determinations are taken into account. It is rather striking that all points are found in or below the same region as in Fig. 2. The deviations are in the direction of lower y/y_0 values. The probable explanation is lack of homogeneity of the reaction mixture, which was also observed. If the bromobenzene goes into solution mainly to the same extent as it reacts, the reaction is forced to consume more tritium than it should if the system were homogeneous, and too low values of y/y_0 will result. The temperature was not controlled in these experiments. Moreover, hydrogen exchange might take some part in the lowering of y/y_0, especially in the experiments of long duration. Exchange, however, is not likely to be the single reason for the lowering of these values (1 b).

A value of $k_T/k_H = 0.55$ at 0° C corresponds to an unusually weak isotope effect if the splitting of a carbon-hydrogen bond is rate-determining, a value of 0.2 or less being more in agreement with theory and experience. On the other hand, the isotope effect is certainly not caused only by secondary effects of the occurrence of heavy hydrogen in the molecule. Accurate determinations of k_D and k_H in the nitration of nitrobenzene-d_5 and ordinary nitrobenzene in sulphuric acid carried out recently by WILLIAMS *et al.* (5) show that the ratio deviates from unity by less than 5 per cent.

The different possibilities for the mechanism of aromatic substitution have been reviewed in an earlier paper (1). If the splitting off of hydrogen is the rate-controlling stage of the reaction, either in a pure S_E2 replacement of hydrogen or in the final decomposition of the intermediate in the two-stage mechanism, the low isotope effect could possibly be explained by the participation of a hydrogen acceptor in the transition state, which tends to increase the zero-point energy of the latter. The fact that there probably is an agreement between the two sets of experiments in the present investigation, and that a comparable isotope effect with deuterium has been observed recently by BRAND (6) in the sulphonation of nitrobenzene-d_5 in strong oleum (30–40 per cent anhydride), however, makes it necessary that the transition state should be the same under these different conditions, for β should be rather sensitive to changes in this state. Even if SO_3 could be proposed as the acceptor common to all experiments, the high value of β still makes the explanation less probable.

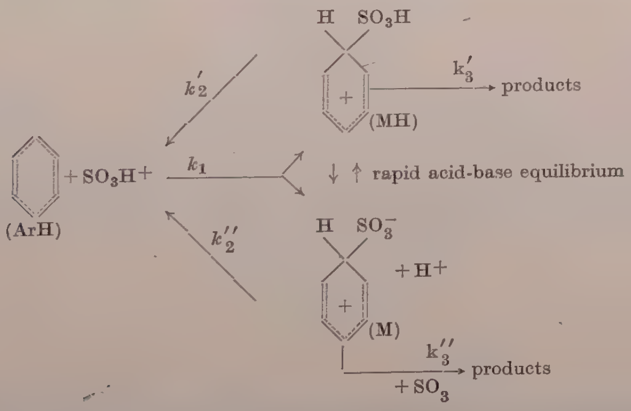
The remaining possibility to explain the low but real isotope effect seems to be that the reaction proceeds according to the two-stage mechanism with the second stage partly rate-determining.

BRAND *et al.* in their recent analysis of the kinetics of aromatic sulphonation in oleum (7) prefer to write the sulphonation reaction simply

$$ArH + SO_3H^+ = ArSO_3H + H^+$$

Owing to the fact that $[SO_3]$ and $[H^+]$ are nearly proportional to one another in oleum (7 c) there remains a certain possibility that S_2O_6 is the attacking entity instead of SO_3H^+. In the following SO_3H^+ is used, but the proportionality mentioned above should be borne in mind in the following.[1]

A general reaction scheme of the detailed mechanism of sulphonation could be given in the following way:



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[1] In the following very simple treatment concentrations are used throughout, the changes in the activity coefficients with medium composition being neglected.

The arguments for the occurrence of the intermediate form M have already been discussed (8, 9) and it is not less probable (1 h) if SO_3H^+ is the attacking agent (7).

The two intermediates MH and M are supposed to be in immediate acid-base equilibrium:

$$\frac{[M]\,[H^+]}{[MH]} = K_a \tag{1}$$

The method of the stationary state then gives

$$k_1[ArH]\,[SO_3H^+] - (k_2' + k_3')\,[MH] - k_2''[M]\,[H^+] - k_3''[M]\,[SO_3] = 0. \tag{2}$$

The experience from nitration makes it very probable that $k_2' \ll k_3'$. Equations (1) and (2) then give

$$v_3' = k_3'\,[MH] = \frac{k_1\,[ArH]\,[SO_3H^+]}{1 + K_a\left(\dfrac{k_2''}{k_3'} + \dfrac{k_3''}{k_3'}\cdot\dfrac{[SO_3]}{[H^+]}\right)} \tag{3}$$

and

$$v_3'' = k_3''\,[M]\,[SO_3] = \frac{k_1\,[ArH]\,[SO_3H^+]}{1 + \left(\dfrac{k_3'}{K_a k_3''} + \dfrac{k_2''}{k_3''}\right)\dfrac{[H^+]}{[SO_3]}} \tag{4}$$

Owing to the proportionality between $[SO_3]$ and $[H^+]$ in the oleum the denominators of equations (3) and (4) will be almost independent of the composition of the medium. It was the desire to get such a result that prompted the proposal of the participation of an SO_3 molecule as acceptor in the final decomposition of M.

Equations (3) and (4) give rise to some interesting conclusions. Since k_2' has been neglected beside k_3', k_2'' cannot be assumed to equal zero, for in such a case the first reaction stage will be completely rate-determining and the total reaction rate equal to $k_1\,[ArH]\,[SO_3H^+]$. The hydrogen mass would have no influence.

If the intermediate is present only in the form M, i.e. $K_a = \infty$, we get

$$v_3'' = \frac{k_1\,[ArH]\,[SO_3H^+]}{1 + \dfrac{k_2''}{k_3''}\cdot\dfrac{[H^+]}{[SO_3]}} \tag{5}$$

Depending on the relative magnitudes of the different quantities the influence of the isotopic mass might be anywhere between none and that on k_3''.

Owing to its possession of a negative charge, the intermediate form M might split off its nuclear hydrogen with such difficulty that k_3'' might be put equal to zero. Equation (3) gives for the corresponding reaction rate:

$$v_3' = \frac{k_1\,[ArH]\,[SO_3H^+]}{1 + K_a\dfrac{k_2''}{k_3'}} \tag{6}$$

The last expression seems to be the simplest explanation of the occurrence of a moderate isotope effect, and the latter should be truly independent of the temporary composition of the medium. It might be that k_2''/k_3'' in equation (5) is more likely to be independent of the particular aromatic molecule in question than $K_a k_2''/k_3'$ in equation (6). Hitherto only a very approximate comparison between the molecules bromobenzene and nitrobenzene has been possible (see above), however, and the agreement should not be overemphasized. It seems difficult to carry the discussion further with the experimental facts available at present.

It might be pointed out, finally, that the well-known differences in the quantitative directing power of substituents between nitration and sulphonation, which have sometimes been ascribed to a difference in the electrophilic nature of the attacking reagent and its sensitivity towards electrostatic influences (7 b), could probably at least partially be explained by the occurrence of constants other than k_1 in the rate expression of sulphonation.

Some of the earlier experiments were carried out at the Nobel Institute of Physics, radio-chemical department, which is supported by Svenska Atomkommittén.

SUMMARY

Sulphonation of bromobenzene containing tracer amounts of bromobenzene-4-t has been carried out with oleum, partly without solvent, and partly in nitrobenzene solution. With oleum strong enough the hydrogen exchange is negligible compared to the sulphonation, the reverse reaction does not occur to any appreciable extent, and it is possible to measure the isotope effect. The experiments in nitrobenzene solution give the value $k_T/k_H = 0.55 \pm 0.09$ at 0° C. In pure oleum lack of homogeneity causes discrepancies, but the value seems to be in reasonable agreement with the same figure.

The bearing of the present result and of some recent results of other authors on the theoretical picture of the mechanism of aromatic sulphonation is discussed.

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5. G. WILLIAMS, private communication.
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Tumorhemmungen

Von Hans von Euler

Mitosegifte

Die Kern- und Zellteilung kann, wie Dustin 1933 entdeckte, durch chemische Stoffe gehemmt werden, und zwar geschieht dies an verschiedenartigen Zellen in verschiedenem Grade. *Stoffe zu finden, welche unter Schonung der normalen Zellen ausschliesslich oder überwiegend die Teilung canceröser Zellen und ihrer Kerne und damit Wachstum von Krebsgeweben hindern, ist eine für die gesamte Krebsforschung ausserordentlich wichtige chemotherapeutische Aufgabe.* Sie liegt durchaus im Bereich des Möglichen — in gewissen Fällen liess sich bei Krebsgeweben ein naher Zusammenhang zwischen Hemmung der Mitosen und des Tumorwachstums zeigen. Andererseits muss aber gleich betont werden, dass eine *allgemein* geltende Beziehung zwischen Hemmung der Tumorwachstums und anti-mitotischen Vorgängen nicht nachgewiesen ist. Die Frage, unter welchen Umständen und innerhalb welcher Grenzen eine solche Beziehung besteht, ist jedenfalls interessant und wichtig genug, um eine Besprechung an dieser Stelle zu rechtfertigen.

Man wird zunächst untersuchen, *welche Körperklassen* imstande sind, einerseits Mitosen, andererseits Krebswachstum zu hemmen, sodann *welche chemischen Vorgänge* bei mitotischen Hemmungen eintreten.

Dem Problem der krebsspezifischen Mitosegifte hat in neuerer Zeit H. Lettré eingehende, besonders an Gewebekulturen durchgeführte Untersuchungen gewidmet, auf die im Folgenden wiederholt hinzuweisen sein wird.

Die Zellteilung kann bekanntlich in zweierlei Weise, entweder durch *Amitose*, oder durch *Mitose* erfolgen, also

1. direkt, durch Abschnürung des genügend grossen, teilungsbereiten Kernes in 2 Kerne und nachfolgende Teilung der zweikernigen Zelle in zwei einkernige: Amitose, oder
2. in 4 Phasen: Nach Chromatin-Abscheidung (Prophase), Auflösung der Kernmembran (Metaphase), Spindelbildung und Scheidung der Chromosomen (Anaphase) erfolgt in der Telophase Abschnürung der Zelle: Mitose.

Bei der mitotischen Teilung findet in der Prophase eine Bildung der nur im Kern vorkommenden Desoxyribo-nukleinsäure statt. Die Umsetzungen derselben machen einen der in chemischer Hinsicht wesentlichsten Vorgänge der Mitose aus. Über den Stoffwechsel der im Zellplasma und in dessen strukturierten Bestandteilen lokalisierten Ribonukleinsäure liegen einzelne Beobachtungen vor, über das Wesen dieser Synthesen und über den Übergang von Desoxyribo- in Ribo-nukleinsäure ist aber noch wenig bekannt.

Setzt man voraus, dass bei der Cancerisierung (Mutation) der normalen Zelle zentrale Stoffwechselvorgänge und die dazu erforderlichen Enzymsysteme verändert werden, so müssen an der Mitose der cancerisierten Zelle andere Enzymbildner und dementsprechend andere Regulatoren (Hemmungsstoffe und Aktivatoren) beteiligt sein als an der Mitose der normalen Zelle.

Man kann annehmen, dass die Zellen *endogene* Regulatoren der Mitose enthalten, und zwar sowohl Aktivatoren als Inhibitoren.

An *exogenen* Regulatoren der Mitose sind bischer nur Inhibitoren (Mitosegifte) systematisch studiert worden.

Die für die Cancerisierung von Zellen verantwortlichen „Duplikanten" (NOTHDURFT) scheinen besonders im Plasma vorzukommen und dort aus „spezifischen praepathogenen" Proteiden zu entstehen.

Nachweis und Messung der Mitosegift-Wirkung

Zur Feststellung einer Mitosegift-Wirkung verwendet man in Teilung begriffene Zellen, an denen ein Hemmungseffekt beobachtet werden kann.[1] Als übersichtlichsten und zugleich empfindlichsten Test empfiehlt LETTRÉ[2] die Gewebekultur embryonaler oder carcinomatöser Zellen. Er benützte besonders in der Gewebekultur gezüchtete Zellen und den Mäuse-Ascites-Tumor, da dieser auch zur morphologischen Untersuchung einer Stoffwirkung sehr geeignet ist.

Zur Methodik der Gewebekultur siehe: R. C. PARKER, Methods of Tissue Culture, 2. ed. New York 1950.

Unter den Untersuchungen, welche an Mäuse-Ascites-Tumoren über die Wirkung eines typischen Mitosegiftes angestellt wurden, sind diejenigen über *Colchicin* in erster Linie zu nennen, und zwar seien erwähnt die Arbeiten von SCHAIRER, von H. BRODERSEN[3], DITTMAR und MAASS[4], sowie aus neuerer Zeit die zahlreichen Beiträge von LETTRÉ und Mitarb.[5].

LETTRÉ und R. KRAPP[5] fanden in Übereinstimmung mit H. BRODERSEN nach Injektion von Colchicin (bis zu einer Dosis von 30 γ) das Maximum der gehemmten Mitosen meist nach 10–13 Stunden. „Wurde die Colchicindosis *über* 30 γ erhöht, so trat das Maximum der Mitosen meist erst später in Erscheinung. Diese Mengen entsprechen schon fast den tötlichen Dosen. Da der Tod nach Colchicininjektionen aber sehr lamgsam eintritt, so gelingt es oft, Versuche in einem Zeitintervall von 24–36 Std. durchzuführen." Das spätere Auftreten des Mitosemaximums deutet LETTRÉ so, dass Colchicin zumindest 2 Angriffspunkte in der Zelle hat. Erst nach teilweiser Ausscheidung von Colchicin, die nach LETTRÉ und LUTZE[6] nach 16 Stunden weitgehend erfolgt ist, kann Teilung und damit Hemmung der Metaphase einsetzen. Die Ergebnisse nach einmaliger Injektion von Colchicin geben LETTRÉ u. Mitarb.[7] in einer Tabelle an (l. c. S. 143), die hier im Auszug folgt. Man erkennt die Abhängigkeit der Mitosenzahl von der Dosis.

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Zahl der Mitosen im Mäuse-Ascites-Tumor nach Colchicin-Inj.

Auszählung von je 1000 Zellen im Ausstrichpräparat (Feulgen-Färbung).

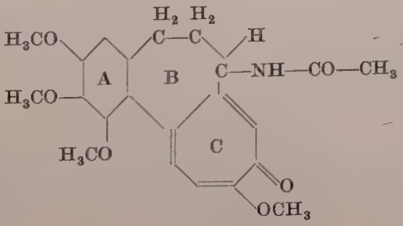
Dosis in γ	Stunden nach der Injektion		
	0	10	24
0	1,5	1,0	1,1
1	1,5	2,2	
3	1	2,4	
5	2,5	4,2	3,2
5	3,3	4,9	4,0
5	2,5	5,4	2,6
7	1.2	8.8	2.9
9	1,2	8,9	2,9
10	3,4	13,6	4,0
10	3,4	10,7	2,1
10	2,0	12,2	
25	2,3	7,1	4,0
30	1,0	0,3	5,0
30	0,7	1,0	16,8
30	1,9	2,2	9,2

Ein Maximum tritt nach 10 Stunden bei etwa 10 γ Colchicin ein (vgl. S. 232). N-Methylcolchicamid, an Hühnerherz-Fibroplasten in der Gewebekultur geprüft, ist ein *stärkeres* Mitosegift als Colchicin. Die untere Grenze der Wirksamkeit liegt für Colchicin bei 0,01 γ/ml, für N-Methylcolchicamid bei 0,0025 γ/ml. Für die Giftwirkung des Colchicins ist nach LETTRÉ die Latenzzeit seiner Wirkung charakteristisch, die auch mit dem 10-fachen seiner sicher tötlichen Dosis nicht verkürzt wird (Z. Krebsf. 57, 148).

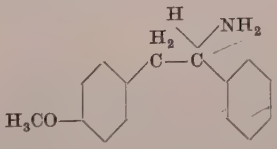
Die Giftigkeit des N-Methylcolchicamids ist bei einmaliger Gabe von der gleichen Grössenordnung wie die des Colchicins; bei mehrfachen Dosen zeigt es kumulierende Wirkung, so dass es doppelt so giftig als Colchicin wirkt. Für die lokale Tumorbehandlung soll es dem Colchicin gegenüber Vorteile bieten.

Struktur des Colchicins; Colchicin-Derivate

Die von WINDAUS 1924 aufgestellte Formel hat durch neuere Untersuchungen, besonders von J. W. COOK[1] und von DEWAR[2] eine Änderung erfahren. Die folgende Struktur dürfte nunmehr, auch durch die Röntgen-Analyse von PEPINSKY, sichergestellt sein.



Colchicin



4′-Methoxy-stilbylamin

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Während alle Abbauprodukte des Colchicins nach Versuchen von LETTRÉ geringere Wirksamkeit zeigen als das Ausgangsmaterial[1] konnte dieser Forscher auf zwei Wegen stärker wirksame Derivate darstellen:

1. durch Erhitzen von Colchicin mit primären und sekundären Aminen, wobei substituierte Colchicamide entstehen[2],
2. durch den Ersatz der Azetylgruppe durch homologe Säuren (LETTRÉ, FERNHOLTZ u. KÖLLING).

In der obigen Strukturformel ist der Ring B des Colchicins ein Siebenring. Auch das 4'-Methoxy-stilbylamin (und zwar nur die linksdrehende Verbindung) ist antimitotisch wirksam. Strukturell analoge Ring-geschlossene und Ring-offene Verbindungen verhalten sich in ihrer anti-mitotischen Wirkung nicht analog. Die von LETTRÉ anti-mitotisch befundenen Derivate der Stilbylamingruppe sind als ein selbständiger Typ von Mitosegiften aufzufassen[3].

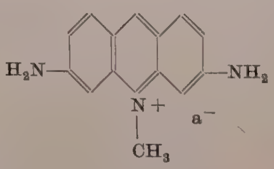
Die folgende Tabelle zeigt die Wirksamkeit einiger Colchicin-Derivate an Hühnerherzfibroplasten (H. LETTRÉ, Scientia Pharm. 1952).

Derivat	Aktivität in γ/ml	Derivat	Aktivität in γ/ml
Colchicin	0,01—0,03	Colchicamid	0,01
O-Metyl-colchicin	0,05	N-Methyl-colchicamid	0,0025
O-Äthyl-colchicin	0,08	N-Äthyl-colchicamid	0,003
O-Propyl-colchicin	0,8	N-Benzyl-colchicamid	3
O-Butylcolchicin	2,5	Colchinol	0,1
Colchicein	4,5	Oxy-colchicin	10

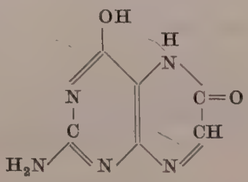
Als Grenze der Colchicin-Mengen, mit denen noch eine erkennbare Wirkung erzielt werden kann, wird ein Wert von über 3 γ angegeben. Bei der Austestung im Gewichtsversuch liegt die untere Grenze einer erkennbaren Wirkung zwischen 2,5 und 5 γ.

Bei Mäusen von 20 g führt die Injektion von je 20 γ Colchicin an 5 aufeinander folgenden Tagen während oder nach dieser Zeit zum Tod der Tiere, die vorher starke Gewichtsabnahme zeigen. Tagesdosen von 10 γ überleben die Mäuse.

Stilbylamin-Derivate. An diesen sind, ebenfalls von LETTRÉ[4], die in nachstehender Tabelle verzeichneten Aktivitäten ermittelt worden:



Trypaflavin



Xanthopterin

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[1] Beim Abbau des dritten Ringes verschwindet die Wirkung. Colchinol ist das einfachste bisher bekannte Abbauprodukt des Colchicins, in dem noch eine Wirksamkeit vorhanden ist.

[2] H. LETTRÉ, Angew. Chem. 55, 265; 1942. Im Colchicamid trägt der Ring C des Colchicins den Substituenten $NH—CH_3$ statt OCH_3.

[3] H. LETTRÉ u. E. HARTWIG, Z. physiol. Chem. 291, 164; 1952.

[4] H. LETTRÉ und J. DELITZSCH, Z. physiol. Chem. 281, 139; 1944. — 289, 220; 1952. — Scientia Pharm. 20, 75 (1952).

Substanz	Aktivität in γ/ml
4′-Methoxy-stilbylamin	4—5
3′-Methoxy-stilbylamin	20
4′-Äthoxy-stilbylamin	0,4
4′-Butoxy-stilbylamin	1,8
4-Methoxy-stilbylamin	unwirksam
4′-Methyl-stilbylamin	10
3′, 4′-Tetramethylen-stilbylamin	10

Diamino-akridine und verwandte Stoffe

3,6-Diamino–akridin-chlormethylat, als *Trypaflavin* bezeichnet, wurde an Fibroplasten[1] und am Mäuse-Ascites-Tumor[2] auf Mitose-Hemmung geprüft. Das 3,6-Diamino-akridin (Proflavin) ist an Hühnerherzfibroplasten ebenfalls noch stark wirksam (2 γ/ml), während das Akridin selbst unwirksam ist. Das Xanthopterin ist ebenfalls als Mitosegift aktiv befunden worden. Lettré zieht aus seinen Versuchen den Schluss, dass Amino-Akridine, Amino-chinoline und -pyridine eine zellteilungshemmende Wirkung haben, „wenn sie in einer tautomeren Diamin-Form auftreten können".

„Wenn wir die Mitosegifte vom Trypaflavin-Typ als Antagonisten der Purine und Pyrimidine in den Nukleinsäuren ansehen" — schreibt Lettré — „so müsste sich daraus die experimentell prüfbare Folgerung ergeben, dass alle Verbindungen, die wir im chemischen Sinne als abgewandelte Purine und Pyrimidine auffassen können, Mitosegifte vom Trypaflavin-Typ sind. Dies ist aber allgemein nicht zutreffend".

Nach der Injektion von Trypaflavin fand Lettré (Z. Krebsf. 56, 7; 1948) ein Absinken der Zahl der Mitosen auf Null und darauf folgende langsame Zunahme zur Norm.

Rondoni und Necco[3] fanden in Akridin-Derivaten mit langen Seitenketten besonders in Atebrin deutliche Mitosehemmungen.

Alkaloide. Lettré[4] hat eine grosse Zahl von Alkaloiden auf ihre Fähigkeit geprüft, die Zellteilung, wie Colchicin, zu hemmen. Dabei ergab sich eine interessante Beziehung zur Stilbylamin-Gruppe: Unter 45 Alkaloiden, die keine Beziehung zum Stilbylamin-System zeigen, befand sich kein Mitosegift; dagegen liessen sich — abgesehen vom Colchicin — noch im Narcotin, Chelidonin, Homo-chelidonin und Methoxy-chelidonin deutliche Wirksamkeiten nachweisen.

Colchicin ruft, wie durch die grundlegenden Untersuchungen von Dustin und von Blakeslee erwiesen wurde, in Pflanzen *Polyploidie* hervor. In welcher Beziehung dieser Effekt zu der mitosehemmenden und der anticancerogenen Wirkung des Colchicins steht, bleibt noch genauer aufzuklären.

Die Erwägung, dass am Polyploidie-Effekt des Colchicins eine Verdrängung der Wuchshormone beteiligt sein könne, haben den Verf. veranlasst, mit A.-M. Perje

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[1] O. Bucher, Z. Zellforsch. 29, 283, 1939. — 30 438, 1940.

[2] H. Lettré, J. physiol. Chem. 271, 192; 1941.

[3] P. Rondoni u. A. Necco, Experientia, 7, 142; 1951. — Siehe auch I. Lasnitzki u. J. H. Wilkinson, Brit. J. Cancer, 2, 369; 1948. — Ferner: Fr. D'Amato, Rend. Accad. N. Lincei, Ser. 8, 612 (1952) u. Caryologia, 2—4 (1951/52) Allium-Test.

[4] H. Lettré und M. Albrecht, Z. physiol. Chem., 281, 133; 1944. — 287, 58; 1951. — Hans Lettré u. Renate Lettré u. Ch. Pflanz, Z. physiol. Chem. 287, 53; 1951.

(Arkiv f. Kemi 20 A No. 2; 1945) den Einfluss des Colchicins in verschiedener Konzentration auf den zeitlichen Verlauf der Keimung durch unseren Keimtest an Siegergerste (Wasser = 10) zu untersuchen und die Chromosomenzahl (normal 2n = 14) zu beobachten.

Lösung	Entwickl.-grad	Chromosomenzahl
Colchicin 0,0001 %	11	Chromosomenzahl normal (2 n = 14).
» 0,05 %	5	Chromosomenzahl 2 n = 28 oder mehr.
» 0,10 %	3	Prophase normal. In der Metaphase sammeln sich die Chromosomen nicht in einer Äquatorialplatte.
Phenylurethan 0,01 %	3	Coleoptilwachstum gehemmt.

Die Injektion von Colchicin in Jensensarkom tragende Ratten hat in diesem Institut noch zu keinen *regelmässig* auftretenden Tumorhemmungen geführt.[1]

Bei unseren früheren Versuchen hatten Ratten von etwa 100 g Körpergewicht mit implantierten Jensensarkomen von ca. 20 g in Zwischenräumen von 2–4 Tagen 2 bezw. 3 mal Injektionen von je 200 γ Colchicin erhalten. Die Tiere vertrugen diese Einspritzungen ohne Schaden und ohne äussere Symptome. Die Hemmung der Wachstumsgeschwindigkeit bei grossen Sarkomen betrug etwa 15%, bei den kleinen Sarkomen über 80%.

Beim Menschen beträgt die Grenzdosis des Colchicins 3–5 mg/Kg.

Lits beobachtete nach subcutaner Injektion von 25 γ Colchicin an Mäusen, dass der Tumor zu wachsen aufhörte und seine feste Konsistenz verlor. In einzelnen Fällen verschwand der Tumor zwei Tage nach der 2. Injektion, in anderen Tieren blieb der Tumor noch nachweisbar. Nach Abnahme des Tumors durch 25 γ Colchicin lebten die Tiere noch 50 Tage.

Suminari Tuboi (Mitt. Med. Akad. Kyoto, 32; 1941) prüft den Einfluss des Colchicins auf die Entwicklung verschiedener Tumoren, u. zw. des Bashford'schen Mäusekarcinoms, ferner eines Rattenhepatoms und eines Kaninchensarkoms. Als letale Dose Colchicin ermittelte er 20 γ per 10 g Mäusegewicht und 100 γ per 100 g Rattengewicht. Er fand das Geschwulstwachstum nach 2 Wochen lang fortgesetzter täglicher Injektion bei Mäusen, Ratten und Kaninchen durch Colchicin deutlich gehemmt.

Colchicin, das, wie die Strukturformel zeigt, mehrere reaktionsfähige Gruppen bezitzt, kann an verschiedenen Zellkomponenten angreifen und kann im Tierkörper mehrere verschiedenartige Giftwirkungen hervorrufen, gegen welche verschiedene Tiere sehr ungleich empfänglich sind (Meerschweinchen z. B. besonders empfindlich).

Cholin bildet einen besonderen Typ der Mitosegifte; selbst im hohen Dosen zeigt es auf Hühnerherzfibroplasten in der Gewebekultur geprüft keine wachstumshemmende Wirkung. „Dagegen konnte am Mäuse-Ascites Tumor durch Injektion von Cholin eine Abwandlung des Mitoseablaufes in Richtung einer Endo-Mitose beobachtet werden“ (Strahlentherapie, 79, 363).

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[1] Auch die Ergebnisse von Brodersen (Strahlentherapie 73, 195; 1943) lassen keine Erwartungen auf eine Heilwirkung bei Hautkrebsen und anderen Tumoren zu.

Mechanismus der Colchicin-Hemmung

Lettré hat versucht, den Reaktionsmechanismus des Colchicins durch synergetisch wirkende Verbindungen aufzuklären. Er[1] fand eine grosse Anzahl von Stoffen, welche die Wirkung des Colchicins an Fibroplasten in der Gewebekultur verstärken. Die Synergisten des Colchicins gehören chemisch den verschiedensten Stoffklassen an, wie die folgende Tabelle (Lettré, Sc. Pharm., 20; 1952) zeigt.

Synergisten des Colchicins

- Tryptamin
- Phlorrhizin
- Aporphin-alkaloide
- Bulbocapnin
- Benzyl-isochinolin-alkaloide
- Papaverin
- Berberin-alkaloide
- Chelerythrin
- Chinin
- Veratrin
- Stilbylamin
- Steroidhormone
- Testosteron
- Östron
- Desoxycorticosteron
- Cortison
- Azetol
- Benzpyren

„Die Alkaloide mit Stilbylamingruppe: Papaverin, Tetrahydropapaverin, Berberin, Tetrahydroberberin und Chelerythrin zeigen an in vitro gezüchteten Fibroplasten eine Verstärkung der Mitosehemmung durch Colchicin. Bei den Opiumalkaloiden Thebain, Codein und Morphin fällt die synergistische Wirkung mit der Abnahme der Krampfgiftwirkung ab.“ . . . „Die Muskelgifte Chinin, Strychnin und Veratrin zeigen verstärkende Wirkung“ (H. u. R. Lettré, Z. physiol. Chem. 287, 150; 1951).

Seine Versuche über die Beziehung zwischen den Dosen von Colchicin + Synergist Phlorrhizin und der Prozentzahl der Mitosen deutet Lettré dadurch, dass er eine Wirkung des Colchicins in dem Komplex der Faktoren annimmt, die zu dem Prozess der Kontraktilität in Beziehung stehen: die Kontraktion der Zellspindel bei der Auseinanderführung der Chromosomen und die Einschnürung der Zelle bei der Teilung werden so in Analogie zur Muskelkontraktion gebracht.[2] Einer von Lettré aufgestellten Arbeitshypothese zufolge ist die mitosehemmende Wirkung des Colchicins veranlasst durch die Hemmung einer Reaktion zwischen Adenosin-triphosphorsäure und einem kontraktilen System von der Art des von Szent-Györgyi[3] aus Muskel isolierten Actomyosins.

Während Lettré die Colchicin-Wirkung als eine Störung der Spindelfunktion betrachtet, führt Ludford dieselbe auf eine Unterdrückung der Spindel-Bildung zurück.

Die nunmehr sichergestellten Befunde, dass durch Colchicin-Injektionen die Tumorentwicklung in tumortragenden Tieren gehemmt wird, dürfte *zum Teil* auf eine Abtötung der Tumorzellen zurückgeführt werden können. Die dabei auftretenden Haemorrhagien sind möglicherweise die Folgen eines Colchicin-Einflusses auf das Enzymsystem der Ascorbinsäure.

Chinone

E. F. Lehmann[4] hat in seinem Testmaterial (Eier einer Ringelwurmart) mittels Benzochinon, Naphtochinon und Phenanthrenchinon sehr starke Zellteilungshemmungen hervorrufen können. Ungeklärt ist noch, auf welchem, vermutlich enzymatischen Weg die sehr reaktionsfähigen Chinone, auf deren enzymschädigenden Einfluss H. Wieland mehrfach hingewiesen hat, antimitotisch wirken. Der Angriff findet wohl an der SH-Gruppe statt, z. B. bei der Succinodehydrase, oder, wie P. Rondoni in Betracht zieht, bei Phosphatasen.

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[1] H. Lettré u. Mitarb., Naturwiss. 37, 378; 563; 1950. — 38, 13, 70; 214, 1951. — Z. physiol. Chem. 287, 53; 150; 1951. — Klin. Wochenschr. 1951, 20.

[2] Siehe auch J. Brachet, Embryologie chimique. Paris 1947.

[3] A. Szent-Györgyi, Chemistry of muscular contraction. New York, 1947.

[4] E. F. Lehmann, Experientia, 3, 223; 1947. Siehe auch C. Dittmar, Z. Krebsforsch. 52, 17; 1942.

Unter den mitotisch wirksamen Chinonen ist hier besonders das *Adrenochrom* zu erwähnen, das ebenso wie das Nor-Adrenochrom unter verschiedenen Umständen im Organismus aus Adrenalin[1] bezw. Nor-Adrenalin (Arterenol) entstehen kann. Mitose-Störungen durch Chinone konnten R. Meier, M. M. Allgöwer[2] u. B. Schär[3] an Fibroplasten feststellen. Sehr bemerkenswert ist die Beobachtung von Kisch[4], dass oxydiertes Adrenalin zwar bei normalen Zellen, aber nicht bei Krebszellen die Atmung erhöht.

Nach allen unseren biochemischen Erfahrungen können Chinone die Krebsbildung in sehr verschiedener Weise beeinflussen. Wir kommen hierauf noch in anderem Zusammenhang zurück.

Hier sei nur darauf hingewiesen, dass Benzochinon als Oxydationsprodukt von p-Phenylendiaminen (den Spaltprodukten cancerogener Azofarbstoffe) auftreten kann.

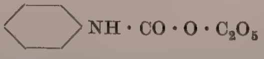
Adrenalin verhält sich bei Hühnerherzfibroplast-Test in der Menge von 100 γ/ml als Mitosegift. Nach Lettré wird diese Wirkung durch Zusatz von Ascorbinsäure oder Glutathion zum Nährmedium aufgehoben. Es soll dies darauf beruhen, dass Ascorbinsäure oder Glutathion „durch Verschiebung des Oxydationszustandes eines Umwandlungsproduktes des Adrenalins dieses als Mitosegift unwirksam macht".

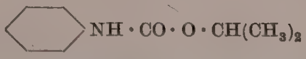
Ascorbinsäure verhindert, mit anderen Worten, die Oxydation des Adrenalins. Der Umstand, dass Adrenalin sich auch in der Krebszelle (Ehrlich'sches Mäusekarcinom in vitro oder als Ascites Tumor) unwirksam erweist, wird so gedeutet, dass in dieser cytochromdefekten Zelle von hohem Reduktionspotential[5] das wirksame Oxydationsprodukt des Adrenalins nicht gebildet resp. reduziert wird.

An reinem Adrenochrom selbst konnte Lettré mitosehemmende Wirkung mit 200 γ/ml nachweisen.

Urethane (Carbaminsäureester)

Deysson[6] hatte 1944 aus seinen Versuchen geschlossen, dass „le phénylurethane et la colchicine sont actuellement les deux corps agissant sur les cinèses de la façon la plus voisine, et cependant . . . il existe de nombreuses différences dans la modalité de leur action". Im Anschluss daran stellten A. Haddow und A. Sexton[7] umfassende Versuche an über die Beeinflussung des Tumorwachstums durch Stoffe, welche den mitotischen Zyklus verändern, und zwar zunächst durch Phenylurethan und Isopropyl-phenylurethan





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[1] Siehe H. Lettré, Z. Krebsf. 56, 5; 1948. — H. Lettré, P. Marquardt u. E. Carl, Z. physiol. Chem. 291, 99 (1952). — Siehe auch F. E. Lehmann, Experientia, 3, 223 (1947).

[2] R. Meier u. M. Allgöwer, Experientia 1, 57; 1945.

[3] R. Meier u. B. Schär, Experientia 3, 358; 1947.

[4] B. Kisch, Biochem. Z. 237, 226; 1931.

[5] B. v. Euler u. H. v. Euler, Dieses Arkiv, 2, Nr. 38; 1950.

[6] G. Deysson, C. r. Acad. Sci. Paris 219, 366; 1944.

[7] H. Haddow u. A. Sexton, Nature 157, 500; 1946.

Phenylurethan und Isopropyl-phenylurethan rufen eine deutliche Verzögerung des Wachstums von spontanem Mamma-Cancer der Maus hervor, und mit beiden Urethanen erhielten Haddow und Sexton Verzögerungen des Wachstums bei Walker Ratten-Carcinom 256.

Man kann diese Wirkung auf die Beeinflussung des Purin-Stoffwechsels zurückführen. Indessen ist auch eine Blockierung der NH_2-Gruppe in Betracht zu ziehen.

Einfluss des Äthylurethans auf das Wachstum des Walker-Carcinoms 16 Tage nach der Implantation.

Kontrollen 12 intraperitoneale Injektionen des Lösungsmittels innerhalb 14 Tagen	Versuche 12 intraperitoneale Injektionen von 50 mg Äthylurethan in Wasser
39,7	8,7
34,8	8,0
32,3	6,7
32,0	4,3
30,9	4,0
27,1	3,9
27,0	3,8
24,2	3,3
22,3	2,9
17,9	0,3
12,0	0,2
5,7	

Schliesslich sind unter den Mitosegiften auch die *Blastocholine* anzuführen, gegen welche Hühnerherz-Fibroplasten sehr empfindlich sind; sie wurden etwa gleichzeitig von Kuhn und Moewus[1] sowie von G. M. und R. Robinson[2] aus Vogelbeeren bezw. aus Malz dargestellt. Sie hemmen das Wachstum gewisser tierischer Gewebe (nicht der Karzinome). Die Blastocholine wurden als Derivate der *Parasorbinsäure* erkannt; sehr wirksam erwies sich auch *Cumarin*. Der von Euler und Mitarb.[3] aus schwarzen Johannisbeeren (*Ribes nigrum*) und *Sorbus aucuparia* gewonnene ,,Faktor J" dürfte zur Gruppe der Blastocholine gehören[3].

Antagonisten der Mitosegifte. Zur Aufklärung des Chemismus der mitotischen Vorgänge können wesentlich beitragen Untersuchungen über die Antagonisten der Mitosegifte, also derjenigen Stoffe, welche den normalen Verlauf der Mitosen aufrecht erhalten oder wiederherstellen. In welche Phase der Mitose ein Stoff eingreift, bezw. wie ein solcher Stoff die amitotische Zellteilung beeinflusst ist meist schwer zu entscheiden. Enzymgifte, etwa Chinone werden sowohl bei mitotischen Vorgängen als auch bei amitotischen Zellteilungen eine Rolle spielen.

Eine der wichtigsten Reaktionen bei der Mitose ist die Bildung der Thymonukleinsäure, deren Polymerisation zu Chromatin durch Trypaflavin gehemmt werden kann (T. Caspersson; J. Brachet).

Die antimitotische Wirkung von Colchicin soll durch Adenosintriphosphat gehemmt werden.

Für die weitere chemotherapeutische Forschung ist die Entscheidung wichtig, ob tumorspezifische Mitosegifte tatsächlich nachgewiesen sind, bezw. ob man schon Anhaltspunkte für das Vorkommen einer solchen Spezifität hat.

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[1] R. Kuhn, D. Jerchel, Fr. Moewus, E. F. Möller und H. Lettré, Naturwiss. 31, 468; 1943.

[2] P. B. Medawar, Lady G. Robinson, Sir R. Robinson, Nature 151, 195; 1943.

[3] Euler, Kem. Arb. Ny Följd B 13, II; 1950. — Euler, H. Söder u. Malmberg, Z. f. Hygiene u. Inf. Krankh. 116, 672; 1935. — Siehe auch Salli Eskola und Solveig Hansen, Suomen Kemistilehti; 1952.

Es ist gewiss möglich, dass sich in Tumoren und in cancerösen Organen besondere Hemmungen geltend machen, etwa zwischen Vitaminen (Co-Enzymen) und Anti-Vitaminen, wie z. B. die zwischen p-Aminobenzoesäure und Sulfanilsäure bestehenden, die um eine enzymatische Gruppe in der Zelle konkurrieren.[1] Solche Fälle sind aber bisher noch nicht genauer bekannt geworden.

Als wesentlichsten Unterschied zwischen den Enzymsystemen eines cancerösen und des entsprechenden normalen Organes hat der Verf.[2] auf Grund seiner mit H. Hellström gewonnenen experimentellen Ergebnisse wiederholt hervorgehoben, dass das Cytochrom-System, nämlich γ-Cytochrom, Cytochrom-oxydase und Diaphorase in cancerösen Organen stark vermindert ist. Die Störung des Cytochromsystems, die sich im Stoffwechsel der Tumoren in hohem Grade geltend macht, wird *bei mitotischen Vorgängen ebenso wie bei amitotischen* eintreten, so dass also auch bei ersteren oxydoreduktive Mitosehemmungen eintreten. Dabei kommen in erster Linie Sulfhydrylstoffe, ferner Nukleinsäuren und ihre Derivate sowie Purin- und Pyrimidin-Nukleotide in Betracht. Auf die verschiedenen Einflusse der Ribonukleinsäure und der Desoxy-ribonukleinsäure kommen wir in anderem Zusammenhang ausführlicher zurück. Es bleiben als wesentliche Fragen zu beantworten: Welches chemische Symptom der eingetretenen Cancerisierung ist in den Mitochondrien das primäre? Ist eine Änderung des Cytochrom-Systemes unmittelbar mit der cancerösen Mutation verbunden, also mit der Einwirkung der Duplikanten, denen, wenigstens zum Teil, ein (enzymatischer) anti-oxydativer (reduktiver) Effekt zukäme.

Stockholm, Universität, Vitamin-Institut u. Institut f. organ.-chemische Forschung.

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[1] Euler, L. Ahlström u. B. Högberg, Dieses Arkiv 17 A Nr. 20; 1943.

[2] Euler, H. Hellström, Z. physiol. Chem. 260, 163; 1939. — 259, 201; 1939.

Tryckt den 3 juli 1953

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ARKIV FÖR KEMI Band 6 nr 19

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Methods of regulating the surface activity and their principal importance for powder metallurgy and powder ceramics

By J. A. Hedvall

With 12 figures in the text

The surface of a solid substance shows characteristics which differ from those of the interior in several respects. At the surface there are particles the affinities of which are wholly or partly unsatisfied. These particles occur especially at the edges and corners of a surface, or where the surface has irregularities. Already on account of their state of energy these particles possess a higher degree of activity towards surrounding media, whether solid, liquid or gaseous, and they also possess a higher degree of mobility.

They are also easier to attack. That was evident already from the earliest observations of the pyrophoric state of metals formed by reduction at low temperatures. Other old-established phenomena can be explained in the same way, for instance why the formation of sulphide starts at the edges and corners of a sheet of silver (Reboul, 1). In neither case is it simply a matter of extension of the attacked surface, which would be correlated to the factor A in the well known expression

$$A \cdot e^{\frac{-q}{RT}}$$

The real effect is connected with a decrease of the exponent q.

In the reverse direction, too, *i.e.* outwards from the surface, it is possible to observe the consequences of the unsaturated state of the particles in the surface. The thermal dissociation of calcite which starts at edges, cracks and corners may be instanced. Perhaps the most pronounced effects of the unsaturated state of chemical affinity may be observed in the adsorption capacity of solid substances, *i.e.* at the formation of the surface layer, which in the case of a possible reaction with the adsorbendum can be regarded as the first stage in the formation of the reaction product (Hüttig, 2).

These effects were first exploited in the chemistry of catalysis. Lowering the energy of activation of the desired reaction through polarization phenomena in the adsorbate layer will primarily increase the rapidity of the catalysed reaction (Langmuir, Taylor, 3).

For powder metallurgy the re-crystallization processes are particularly interesting. In this connection it shall only be pointed out that every disturbance in the interior has an influence on the activity of the surface. Those disturbances may consist *e.g.* of hereditary and transition states in the imperfections of build, or may be caused by alien atoms in the lattice; they exert an influence on the rapidity of the re-crystallization process and on the strength of the substance formed by compression o spontaneous close-packing in sintering.

Researches on ferric oxide, with and without imperfections of build, gave th earlier instances of the influence of these imperfections. The commencement of a reaction, *e.g.* reduction or oxidation, made a considerable difference in the strengtl of the pastilles (Hedvall, 4). Crhistensen and Calbick (5) as well as Shaler an Wulff (6) recently investigated the sintering process in various materials wit different methods and obtained similar results.

It was found later that gases which had been dissolved in the surface. influence not only the rapidity of the re-crystallization process, but also the quality of th active centres (Hedvall, Günther, 7).

Even though metal powders were not used in these experiments the results ca be applied to metallic systems also, as Schwarzkopf has pointed out in his well know works on powder metallurgy.

Generally speaking we may say that many methods used in industrial practic must be far more closely studied with respect to their effects in detail and wit elimination of all disturbing secondary factors. In other words: it is unavoidable t study separately each and every factor which exerts an influence on the activity o the surface. And it is also necessary to exploit to a far greater extent than now th results obtained from *mineralography* about the possibilities to change the activit of surfaces. This is particularly true of powder metallurgy and its sister discipline ceramic powder processes, and the fact has received due prominence at the International Congress for Powder Metallurgy in Graz in 1948, and also at the International Symposium for the Reactivity of Solids in Gothenburg 1952.[1]

But not only in its relation to surrounding media does the surface and its properties compel our interest. As every attack on a substance must start at a surfac this interest is evident. But the very *structure* of the surface layer shows considerabl difference from that of the interior. This difference is also caused by the unsaturate state of energy of the surface particles; it is known that the distances between th particles in the surface differ from the distances in the interior, and in consequenc there are formed groups of "surface molecules" or particles at the surface (Zwicky, & Goetz, 9; Fricke, 10). Such molecular groups play important rôles for every kin of processes at the surface. Lenard-Jones has shown that the first stage of the dissolution of a metal in an acid consists of the detachment of small blocks. Peculia processes, involving transformations of the surface structure in metals, without an corresponding changes in the interior, have also been observed (Cohen, 12) and thei chemical effects have been studied (Hedvall, 13).

Investigations about the migratory capacity of particles at phase boundarie possess a particular value for every process where it is a question of diffusion, re-crystallization, sintering or decomposition. It has already been mentioned that th energy required for detaching such particles is smaller than in the case of those inside the lattice. Nowadays we are therefore not surprised that only the component of the lattice but also adsorbed molecules are able to migrate with a high degree c rapiditly along surfaces or phase boundaries, and this holds true even with larg organic molecules (Volmer, 14). The fundamental importance of this type of transpo of matter has been demonstrated both for catalytic processes (Cassel, 15; Stranski

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[1] The conferences and communications will be issued 1953.

16), and for reactions in general.[1] FRENKEL has published some interesting studies on the mobility of surface particles (17).

Even the first instances of reactions in powders and the so-called place-change reactions (HEDVALL and HEUBERGER, 18), made the migratory capacity of molecular groups appear probable. The transport of matter in the form of such large complexes, *i.e.* solely by diffusion in the lattice, does not appear probable in undisturbed lattices. Even in otherwise undamaged lattices where there is a high concentration of imperfections, *e.g.* AgJ and HgJ, the reaction product (Ag_2HgJ_4) is not formed with any great rapidity in so far as the formation is caused by ionic diffussion in the lattice (WAGNER, 19).

This type of transport of matter is probably of little practical importance, whether the reactions refer to powder metallurgy, ceramic powder processes or silica or oxide chemistry. It is here rather a question of migration of ions or molecular groups at surfaces, particle boundaries or lattice canals (ZIMEN, 20). The migration of molecular groups was recently demonstrated by experiment (JAGITSCH and LINDNER, 21). It might perhaps be repeated once more that the hypothesis of "surface molecules" with a special migratory capacity does not imply any peculiar thing. It is only one of the many abnormalities which occur at the surface of ionic lattices (DE BOER, 22). Moreover, we must also count on local and temporary appearances of molecular groups in the interior of such lattices, groups which are formed through polarization of colliding ions (HEDVALL, 23). In this connection a word of caution should be put forward against far-reaching conclusions from the present knowledge of lattice structure at normal temperatures. Very little is known about the changes in the structure on heating, but, in principle, it must be evident that fundamental changes take place, and that these changes exert a decisive influence on the interior and exterior qualities of the crystals.

There should also be a mention of the important work done by SCHWAB (26), bearing on the question of the relation between lattice structure and activity in a catalyst (ADADUROV, 24; BALANDIN, 25). It was possible to establish a connection between the activity of the catalyst and the concentration of valency electrons at a reaction in which different but comparable alloys were used as catalysts (HUME-ROTHERY).

It will be evident from the preceding brief survey that every disturbance of the equilibrium within a lattice, whatever the type or the magnitude of the disturbance, must influence the activity of the surface.[2] This fact is interesting also to the industrialist after it had been shown that solid substances are not only attacked from outside, as was believed earlier, but that they themselves also can attack surrounding substances, whether solid, liquid or gaseous (HEDVALL, 27).

The practical application of these researches is closely connected with the irreversible phenomena of the lattice, whether they are structural defects or pure energetic disturbances. It is possible to create and regulate such meta-stable transition states and phases by various methods, and the possibility therefore exists to change the physico-chemical activity of a solid substance in a way that will suit our purposes. If the aim be durability, active states must be avoided. If, on the other hand, the intention is to bring it to react completely and rapidly, or to use the substance as an adsorption medium or a catalyst, it is nowadays possible to subject the substance to some treatment to make it suitable to such a purpose. In the case of powder metallurgy and powder ceramics the interaction between the grains is of paramount importance, and consequently also all the circumstances which stem from that factor.

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[1] The transport of reacting or migrating particles in the form of lattice diffusion or surface migration has recently been studied in systems of oxides by HEDVALL, LINDNER and their collaborators cf. J. A. HEDVALL, Einführung in die Festkörperchemie (Vieweg, Braunschweig 1952).

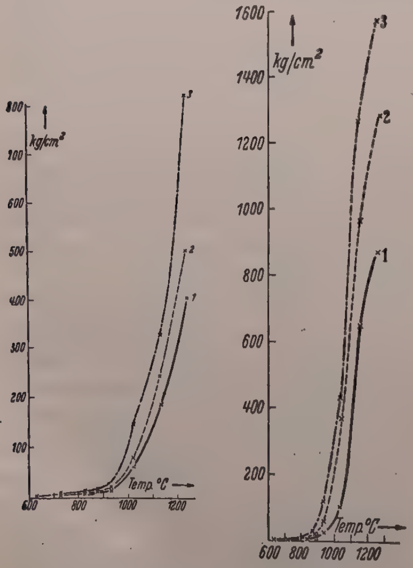
[2] Cf. J. A. HEDVALL. Einführung in die Festkörperchemie (Vieweg, Braunschweig 1952).

With regard to metals the science of metallography has for quite a time investigated such questions, studying the interconnections between interior structure, surface treatment, and chemical, physical or mechanical properties. Corresponding investigations in the case of non-metallic substances were started (Hedvall) only in the early years of the present century. Already the earliest proven reactions in powder mixtures showed clearly the influence of provenance, of defects in the crystallographic formation, and of unstable transitional states. It was consequently clear that there was a need of a science of *mineralography*, corresponding to that of metallography. The lines of research were gradually laid out, and the results have been important for metallographical theory also; we shall only mention here the influence of magnetic, electric and irradiation states (Hedvall, 28).

Our researches on the influence of disturbances in the structure or in the state of energy of the lattice on the physico-chemical properties of the surface resulted in the discovery of a number of previously unknown effects and new types of reactions. The two groups of phenomena not only provided the foundations of mineralography, but also gave many important sidelights on metallography.

We shall primarily treat the following types of effects, and for the sake of simplification adopt a table form with diagrams.

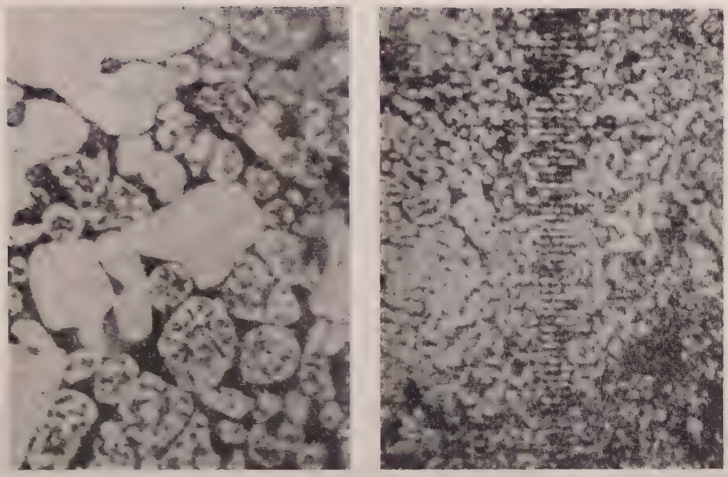
I. The influence of real changes in structure on recrystallization, sintering, reactions between solids, reduction and oxidation



1 a

1 b

Fig. 1. Sintering and shrinking of ferric oxide pastilles: *1 a:* ferric oxide without hereditary defects; curve (1) only heated; curve (2) reduced to Fe_3O_4; curve (3) subjected to reduction and re-oxidation. *1 b:* Ferric oxide from sulphate with hereditary defects; curve (1) only heated; curve (2) reduced to Fe_3O_4; curve (3) subjected to reduction and re-oxidation.



2 a 2 b

Fig. 2. Formation of alloys and re-crystallization take a more rapid course when the oxides in question (in this case CoO and NiO) occur as solid solutions and are reduced (2 a) than when they are merely mechanically mixed (2 b) (HEDVALL and CASSIRER-BÁNO, 29).

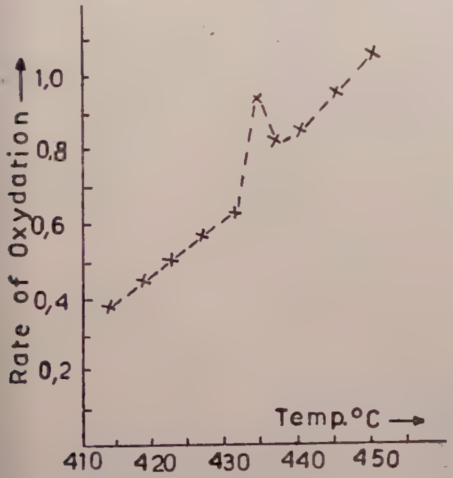


Fig. 3. The oxidation velocity (ordinates) of an alloy (in this case AgCd) attains a relative maximum at the transition point (433° C.) (HEDVALL and ROSÉN, 30).

Temperature in °C	400	450	500	550	600	650	675	700	800	900
Reacted CaO in percent of the total amount present in CaO + Cu_3Sn + O_2	0,0	0,0	0,0	0,0	0,0	1,5	5,2	6,0	6,2	8,1
Reacted CaO in percent of the total amount present in CaO + SnO_2 . .	0,0	0,0	0,0	0,0	0,0	0,0	0,0	2,9	3,0	8,3

Fig. 4 (table). Rapid reactions occur in mixtures of CaO and Cu-Sn alloys, and calcium stannate is formed in the transition intervals (for Cu_3Sn 600 to 650° C.) (HEDVALL and ILANDER, 31). This takes place according to the generally valid formula $M'O + M''X + O_2 \rightarrow M'XO_n + M''O$; where M′ and M″ are metals and X a metalloid (*e.g.* a halogen, carbon, sulphur, phosphorus, silicon, tin). For comparison one may observe the considerably higher reaction temperature for mixtures between CaO and SnO_2.

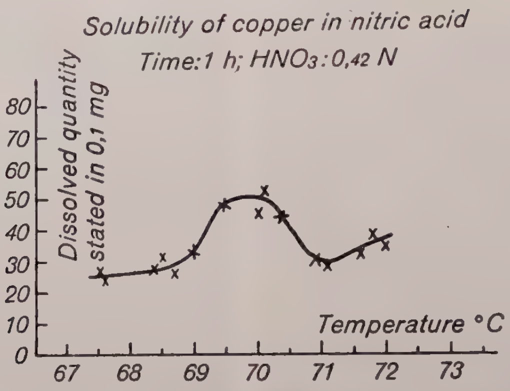


Fig. 5. Cohen has shown (32) that in the case of block structure there are also peculiar changes in the structure of the surface. The rule that a solid substance attains a relative maximum of reactivity at a transition holds also in such cases (here Cu), Hedvall (33).

II. Influence of changes in the magnetic state without any change of modification in the lattice

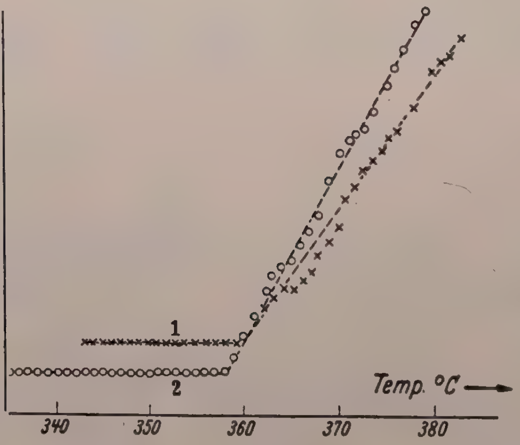


Fig. 6. Change in the catalytic effect (ordinates) at the loss of ferro-magnetism in the reaction $N_2O \rightarrow N_2 + O_2$ with pure nickel as catalyst (Curie interval 358–361° C.). The two curves refer to slightly different velocities of the gas currents (Hedvall, 34).

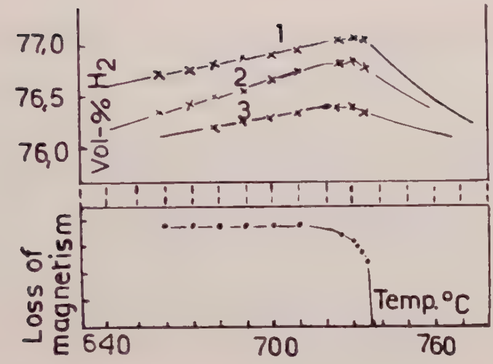


Fig. 7. Change in the catalytic effect at the loss of ferro-magnetism in the reaction $CO + H_2 \rightarrow CH_4 + H_2O$ with cast iron as catalyst. The diminishing percentages of hydrogen above the Curie temperature (in this case about 730° C.) correspond to the increased formation of CH_4. The lower curve depicts the gradual loss of ferromagnetism (35).

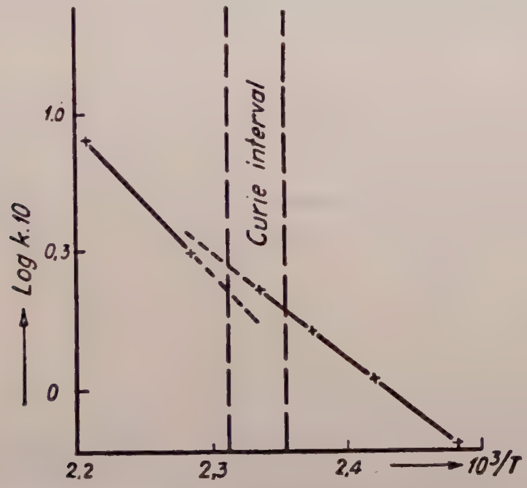


Fig. 8. Kinetic experiments have been made with the decomposition (ordinates) of formic acid, using nickel as catalyst. These show an increase in the catalytic effect in the para-magnetic state compared to the ferro-magnetic state, and this phenomenon is caused by a considerable increase in the number of active centres at the transition from the ferro-magnetic to the para-magnetic state (GÜNTHER COHN, 36). Recent investigations, carried out by JUSTI and VIETH (unpublished as yet) have shown the importance of the electron exchange between substrate and catalyst and its depending on the magnetic qualtities.

III. Influence of spontaneous polarization reversals in the electrical state of the molecules

Such results mean interesting advances in magneto-chemistry and electro-chemistry. Creation of exterior magnetic or electric fields, which can be changed in several respects, will in all probability have an effect on the state of the surface, but have so far not been fully experimentally examined.

IV. Influence of electro-magnetic and supersonic radiation on the active centres of the surface

Experiments with a coherer tube, of a type used in the childhood of wireless, showed that the electric waves had a marked influence on the activity of the nickel powder. This phenomenon is connected with the formation of linkages between the nickel grains and therefore with changes in their maximum reactivity.

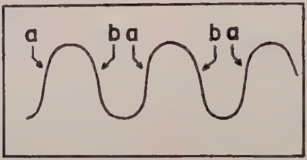


Fig. 9. The figure shows the influence on the yield curve (ordinates) for the reaction $N_2O \rightarrow N_2 + O_2$, when the catalyst (Ni) in a coherer is periodically exposed to radiation from electrical discharges, the temperature being kept constant. (HEDVALL, AHLGREN, 38.)

It might be expected that subjection of metals to supersonic radiation would facilitate the formation of tarnishing layers. In a series of experiments with copper, brass and steel in various gases this hypothesis was confirmed. — The influence of supersonic waves is not unknown in powder metallurgy, but the experiments have generally been made in such a way that there is some doubt whether the effect is caused by temperature rises or solely by the supersonic waves.

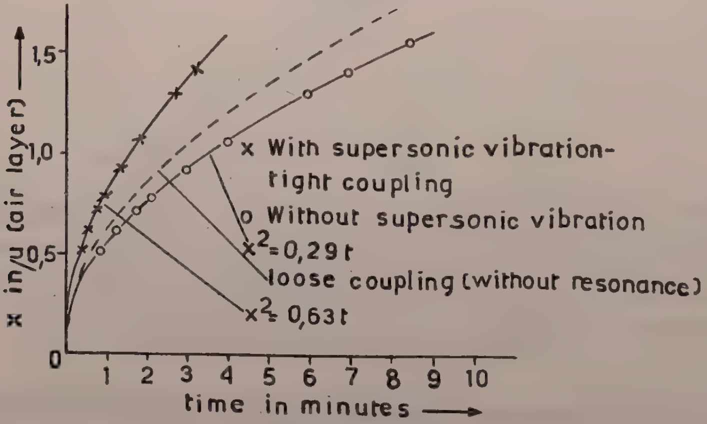


Fig. 10. The three curves show the tarnishing progress on copper rods. with (upper curves) and without [lower curve] coupling to the quartz sender (about 300 kH). The divergent courses of the curves indicate the facilitating influence of the supersonic waves on the formation of tarnishing layers. The rods were kept at constant temperature, and it was proved by kinetic experiments that the effect did not have a thermal origin, but was caused by the influence of the vibratory state on the formation of nuclei and on the diffusion at the surface. (HEDVALL, DAHR, EKWALL, 39.)

V. Influence of dissolved or adsorbed gases

Several recent investigations on non-metallic substances (oxides, salts, etc.) have shown that gases which cannot be considered active in the usual sense of this term, are dissolvable in solid substances (*e.g.* nitrogen and oxygen in silica, alumina, ferric oxide, glass) and influence their surface activity to a marked degree (HEDVALL, 40; HÜTTIG, 41).

These observations include determinations of changes in the surface tension, in the re-crystallization process and in the transition and reaction velocities. These results are naturally highly interesting for powder metallurgy (though they have been far from fully exploited), particularly where materials are made directly by reduction of oxides. Systematic researches on these problems have been made by TIKKANEN (42).

The dissolved gases disturb the lattice and influence the surface potential and consequently every process which takes place at the surface. The influence of gases must therefore be intensively studied, particularly in the cases where sintering, re-crystallization and diffusion play a rôle. These problems are especially urgent in the case of powder metallurgy and thereotically and practically analogous powder ceramic processes: the problems of jet propulsion in aeroplanes may serve as a single example.

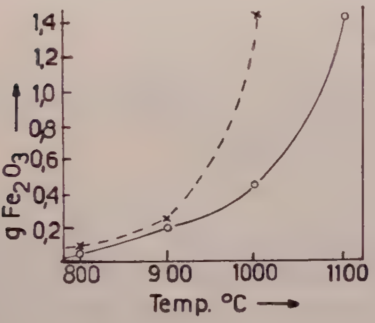
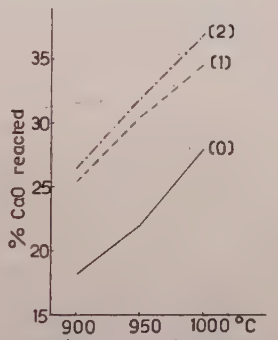


Fig. 11.



Reactivity of tridymite after preheating in O_2 at 600° (1) and 900° (2) and in air (0).

Fig. 12.

Fig. 11. Changes in the adsorption activity of ferric oxide after heating in oxygen [Curve (o)] and in nitrogen [curve (x)]. The quantities of ferric oxide required for adsorption of a fixed quantity of the adsorbate are shown on the abscissa. It will be seen that the adsorption capacity of a ferric oxide preparation declines more rapidly if it has been preliminarily heated in nitrogen than if it has been heated in oxygen. (HEDVALL, GÜNTHER 7.)

This rapid survey of some comparatively new results, which already have been exploited extensively and started large and deep scientifical work, may now be concluded. There is no doubt that these results which form the basis of a new branch of research, *mineralography*, can give many valuable impulses also to the elder sister science; together they may form a new science, more profound and more richly facetted.

The systematic exploitation of the possibilities to regulate surface activity is evidently important in the case of powder metallurgy and ceramic powder processes, and in every kind of process which is founded on sintering, re-crystallization, and reactions where one or more of the partners are powders.

The production of a large number of devices in *e.g.* modern electronics and air plane technics is fundamentally depending on regulating the activity of metals and non metallic substances.

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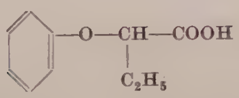
Stereochemical studies on plant growth regulators. V.[1]

Some optically active α-aryloxy-*n*-butyric acids

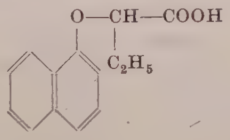
By MAGNUS MATELL

With 9 figures in the text

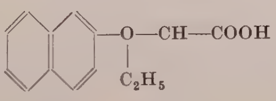
The establishment of the steric relations of some optically active α-aryloxypropionic acids has shown that those of the D-series are more effective as auxins than the corresponding L-compounds (1–4). It is most probable that similar relations are valid for the homologous *n*-butyric acids. To verify this α-phenoxy-*n*-butyric acid (I) and the two isomeric α-naphthoxy-*n*-butyric acids (II, III) were resolved into optical antipodes, which were finally sterically connected to the propionic acids using the method of quasi-racemates (5, 6). At first sight the investigation of other growth regulators more dissimilar to the propionic acids may seem more interesting. However, in the first place the possibility of solving a steric problem by the method of quasi-racemates is rapidly decreasing with increasing structural differences between the compounds to be compared and in the second place it seems interesting to follow the alterations of the biological effects when ascending the homologous series.



I



II



III

In a previous paper (1) the use of WINTHER's rule (7) for the establishment of steric relations was discussed and it was concluded that this rule may be applied to structurally similar acids. With cinchonine the (+)-forms of α-(1-naphthoxy)-propionic acid and α-(1-naphthoxy)-*n*-butyric acid give more sparingly soluble salts than the (−)-forms. Thus, according to the modified WINTHER's rule, the acids with the same direction of optical rotation probably have the same configuration.

By comparing the rotatory powers of optically active compounds in different solvents it is sometimes possible to find their steric connections. In Table 1 some data for (+)-α-(1-naphthoxy)-propionic acid [(+)-1-NOP] have been arranged to give an increasing series. It is seen that the values for (−)-α-phenoxy-*n*-butyric acid [(−)-I] and (+)-α-(1-naphthoxy)-*n*-butyric acid [(+)-II] give increasing series too and thus indicate the same configuration as (+)-1-NOP, while the data for the 2-naphthoxy acid give no informations on the steric relations.

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[1] Part IV: Ark. Kemi *5*, 379 (1953).

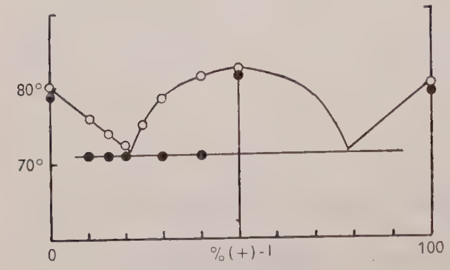

Fig. 1. α-Phenoxy-*n*-butyric acid (I).

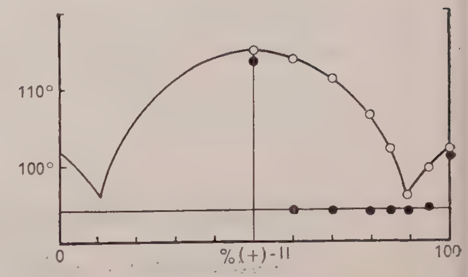

Fig. 2. α-(1-Naphthoxy)-*n*-butyric acid (II).

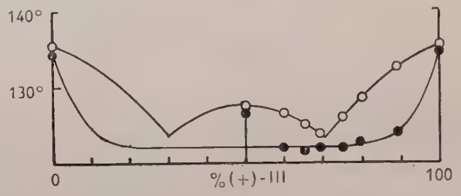

Fig. 3. α-(2-Naphthoxy)-*n*-butyric acid (III).

Table 1. $[\alpha]_D$ for (+)-α-(1-naphthoxy)-propionic acid [(+)-1-NOP], (−)-α-phenoxy-*n*-butyric acid [(−)-I], (+)-α-(1-naphthoxy)-*n*-butyric acid [(−)-II] and (+)-α-(2-naphthoxy)-*n*-butyric acid [(+)-III] in different solvents.

Solvent	$[\alpha]_D$			
	(+)-1-NOP	(−)-I	(+)-II	(+)-III
Acetone	+ 47.2°	− 65.5°	+ 5.0°	+ 97.6°
Ethanol	+ 49.6°	− 51.4°	+ 6.6°	+ 91.8°
Chloroform	+ 58.8°	− 36.4°	+ 9.6°	+ 61.6°
Water (Na-salt)	+ 95.9°	− 20.6°	+ 46.8°	+ 67.3°
Benzene	+ 149.6°	− 5.2°	+ 94.2°	+ 84.1°

By means of the contact method (8), mixtures of the actual *n*-butyric acids with the structurally similar propionic acids described in previous papers (1–4) were studied. Molecular compounds were only formed between homologues with opposite directions of optical rotation, and to get more evidence for the quasi-racemic nature of these compounds, thaw-melting point curves were determined.

From Fig. 1–3 it is evident that the racemic butyric acids are true racemates. However, the tendencies of the antipodes to form racemic compounds, as measured by the extent of the middle branches of the curves, are small, except for the the 1-naphthoxy-acid, which may explain why molecular compounds were found only in mixtures of homologues.

The melting point diagram of (+)-α-phenoxy-propionic acid and (−)-α-phenoxy-*n*-butyric acid (Fig. 4) shows the existence of a molecular compound in the ratio 1:1. The quasi-racemic nature of this is confirmed by the dissimilar diagram of the acids with the same direction of rotation (Fig. 5). The latter system may be classified as type V according to ROOZEBOOM, the components showing far reaching solubility

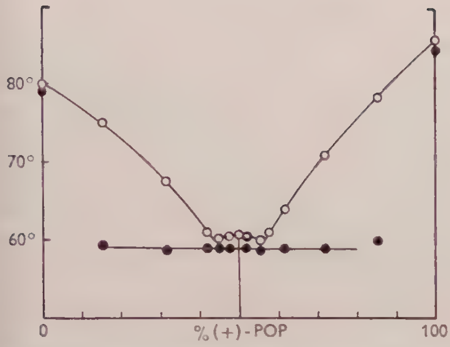

Fig. 4. (−)-α-Phenoxy-propionic acid (POP)
and (+)- » -n-butyric »

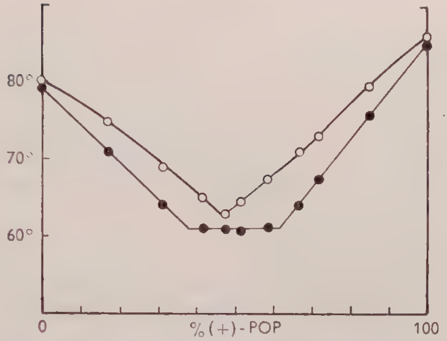

Fig. 5. (+)-α-Phenoxy-propionic acid (POP)
and (+)- » -n-butyric »

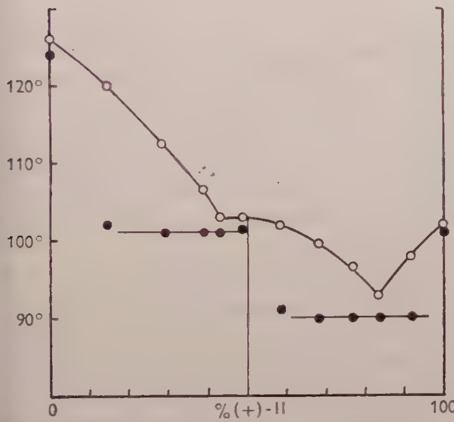

Fig. 6. (−)-α-(1-Naphthoxy)-propionic acid
and (+)- » -n-butyric » (II)

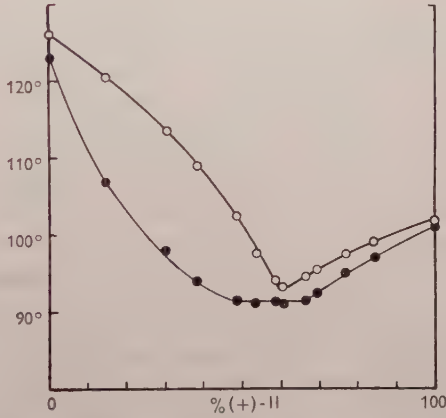

Fig. 7. (+)-α-(1-Naphthoxy)-propionic acid
and (+)- » -n-butyric » (II)

in the solid state. It is thus proved that the *phenoxy-acids with the same direction of rotation* belong to *the same steric series.*

Similar conditions prevail in the systems of naphthoxy-acids as can be seen from Fig. 6–9. However, the molecular compounds are manifested through more pronounced maxima (Fig. 6 and 8). As there is no doubt about the quasi-racemic nature of the molecular compounds in the latter systems, their components must have opposite configurations.

The facts discussed here, especially the formation of quasi-racemates, unequivocally prove that the optically active acids I–III have *the same configuration* as the homologous propionic acids with *the same direction of optical rotation.* According to FREDGA and MATELL (+)-α-phenoxy-, (−)-α-(1-naphthoxy)- and (+)-α-(2-naphthoxy)-propionic acids are D-compounds (1, 2); the homologous n-butyric acids consequently should be written D-(+)-I, D-(−)-II and D-(+)-III.

The biological effects of these compounds are under investigation by Dr. B. ÅBERG, who has kindly put some preliminary results at the author's disposal. In general their effects are very similar to those of the corresponding propionic acids. Thus the

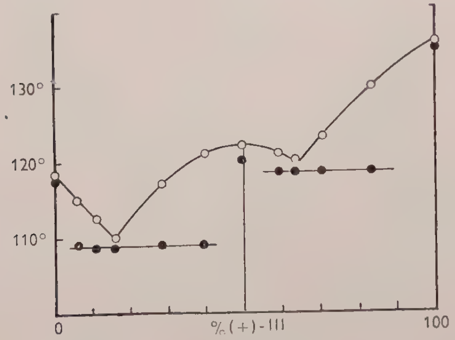

Fig. 8. (−)-α-(2-Naphthoxy)-propionic acid and (+)- » -*n*-butyric » (III)

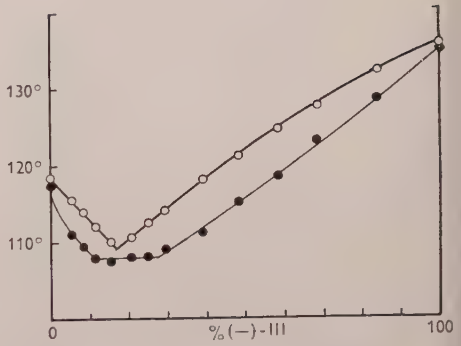

Fig. 9. (−)-α-(2-Naphthoxy)-propionic acid and (−)- » -*n*-butyric » (III)

(+)-form of α-phenoxy-*n*-butyric acid is about 65 times more effective than the (−)-form in suppressing root growth while (+)-α-(2-naphthoxy)-*n*-butyric acid, which is a rather strong auxin, is about 800 times more effective than the (−)-acid in the same respect. For the enantiomorphic 1-naphthoxy-acids the difference is very small; in this case the (+)-acid is the less active. The essential point is that the D-forms are more active than the corresponding L-forms and thus the butyric acids investigated here constitute further instances of the stereochemical specificity, which seems to be a general property of compounds of this type.

Recently LEAPER and BISHOP have suggested that the growth regulating power of aryloxyalkylcarboxylic acids depends on the ability of the aromatic part of the molecule to form a quinonoid structure (9). There seems to be no direct experimental evidence for such a reaction neither *in vivo* nor *in vitro*. However, in the course of this investigation it was found that the naphthoxy-butyric acids are unstable in solution, yielding strongly coloured products. Some preliminary experiments have shown that the product of decomposition of the 2-naphthoxy-acid shows strong absorption in the same region as 1.4-naphthaquinone. A similar instability has also been observed during the preparation of a few other aryloxycarboxylic acids and in order to find out if this phenomenon may be connected with the theory mentioned above, this problem will be further investigated.

Experimental

Racemic α-aryloxy-n-butyric acids (*I–III*). The optically inactive acids, which have already been described in the literature (10, 11), were prepared in good yields by hydrolysis of the esters obtained by refluxing equimolecular quantities of ethyl α-bromo-butyrate and the appropriate sodium phenolates in abs. alcohol. Further data are found in Table 2.

Optically active α-phenoxy-n-butyric acid (*I*). In preliminary experiments crystalline salts were obtained with cinchonine, quinine and strychnine. These salts yielded levorotatory acids but only the salt with strychnine gave a product with an appreciable rotatory power. As no crystalline products yielding the (+)-form of the acid were obtained when using the racemic acid further attempts were made with a pro-

Table 2. α-Aryloxy-*n*-butyric acids

α-Aryloxy-*n*-butyric acid	Recrystallised from	M.p.		Equiv. wt.	
		lit.	found	calc.	found
α-Phenoxy-	Petroleum (b.p. 60–80°)	82– 83° (10)	81.5– 83.3°	180.2	180.4
α-(1-Naphthoxy)-	Cyclohexane and petroleum (b.p. 60–80°)	113–114° (11)	113.5–115.3°	230.3	228.9
α-(2-Naphthoxy)-	Ethanol (50 %) and petroleum (b.p. 60–80°)	126.5° (11)	125.5–127°	230.3	231.0

duct containing about 85 % of the dextrorotatory acid. Under these conditions the morphine salt crystallised and on decomposition yielded almost pure (+)-acid.

α-Phenoxy-*n*-butyric acid (22.6 g, 0.125 mole) and strychnine (42.0 g, 0.125 mole) were dissolved in hot 25 % alcohol (400 ml). On cooling the salt crystallised and was collected after standing over night in a refrigerator. The salt was recrystallised as seen from Table 3; $[\alpha]_{\mathrm{D}}$ refers to the rotatory power of the acid in abs. alcohol.

Table 3. Recrystallisation of the strychnine salt of α-phenoxy-*n*-butyric acid.

Cryst. No.	25 % ethanol (ml)	Weight of salt (g)	$[\alpha]_{\mathrm{D}}$ of the acid
1	400	37.0	−29.9°
2	320	27.4	−43.4°
3	240	25.2	—
4	240	23.2	−46.5°
5	240	21.1	−46.1°
6	240	18.7	−48.3°
7	240	17.0	−49.0°
8	240	15.2	−49.1°

The pure strychnine salt was treated with aqueous ammonia, the alkaloid removed and the residue acidified when the organic acid was precipitated as colourless crystals. The crude acid (5.3 g), m.p. 79–80°, was recrystallised twice from petroleum (b.p. 60–80°). 3.7 g of the pure (−)-acid were collected as small colourless needles, m.p. 79.3–80.3°.

78.75 mg acid: 9.05 ml 0.04823-*N* NaOH
$C_{10}H_{12}O_3$ calc. equiv. wt. 180.2
found » » 180.4

0.0810 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_{\mathrm{D}}^{25} = -0.416^\circ$. $[\alpha]_{\mathrm{D}}^{25} = -51.4^\circ$; $[M]_{\mathrm{D}}^{25} = -92.6^\circ$.
0.1035 g dissolved in *acetone* to 10.00 ml: $\alpha_{\mathrm{D}}^{25} = -0.678^\circ$. $[\alpha]_{\mathrm{D}}^{25} = -65.5^\circ$; $[M]_{\mathrm{D}}^{25} = -118.0^\circ$.
0.1072 g dissolved in *chloroform* to 10.00 ml: $\alpha_{\mathrm{D}}^{25} = -0.390^\circ$. $[\alpha]_{\mathrm{D}}^{25} = -36.4^\circ$; $[M]_{\mathrm{D}}^{25} = -65.6^\circ$.
0.1087 g dissolved in *benzene* to 10.00 ml: $\alpha_{\mathrm{D}}^{25} = -0.056^\circ$. $[\alpha]_{\mathrm{D}}^{25} = -5.2^\circ$; $[M]_{\mathrm{D}}^{25} = -9.3^\circ$.
0.0844 g neutralised with *aqueous sodium hydroxide* and made up to 10.00 ml: $\alpha_{\mathrm{D}}^{25} = -0.174^\circ$. $[\alpha]_{\mathrm{D}}^{25} = -20.6^\circ$; $[M]_{\mathrm{D}}^{25} = -37.2^\circ$.

The mother liquids from the recrystallisations 1 and 2 (Table 3) gave an acid containing about 85 % of the (+)-form. This acid (10.8 g, 0.060 mole) and an equimolecular quantity of morphine (18.2 g) were dissolved in 150 ml of hot 20 % ethanol. After cooling to room temperature the solution was seeded with crystals from a preliminary run and allowed to stand in a refrigerator over night. The solid was collected, the acid liberated from a small sample and the rotatory power determined in abs. alcohol. As seen from Table 4 only three recrystallisations were necessary for the purification of the salt.

Table 4. Recrystallisation of the morphine salt of α-phenoxy-*n*-butyric acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	[α]D of the acid
	Water	Ethanol		
1	25	125	17.2	+48.6°
2	30	100	16.4	+49.2°
3	30	100	12.9	+49.7°

The organic acid was isolated from the pure morphine salt and the crude acid (3.5 g) recrystallised from petroleum (b.p. 60–80°) yielding 3.05 g of the pure (+)-acid. M.p. 79.2–80.2°.

64.70 mg acid: 7.42 ml 0.04823-*N* NaOH
$C_{12}H_{10}O_3$ calc. equiv. wt. 180.2
found » » 180.8

0.1077 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{25} = +0.551^\circ$. $[\alpha]_D^{25} = +51.2^\circ$; $[M]_D^{25} = +92.2^\circ$.

Optically active α-(1-naphthoxy)-n-butyric acid (II). With cinchonine, cinchonidine and strychnine crystalline salts were obtained, which on decomposition yielded products containing excess of the (+)-acid. The best resolution was obtained with cinchonine.

α-(1-Naphthoxy)-*n*-butyric acid (19.8 g, 0.086 mole) and cinchonine (25.4 g, 0.086 mole) were dissolved in hot alcohol (300 ml), water (350 ml) was added and the solution left to crystallise at + 4° C till next day when the solid was collected. The puri-

Table 5. Recrystallisation of the cinchonine salt of α-(1-nahpthoxy)-*n*-butyric acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	[α]D of the acid
	Water	Ethanol		
1	350	300		
2	260	320	25.6	+50.4°
3	250	350	18.0	
4	175	175	14.2	+86.7°
5	135	135	13.0	+94.2°
6	135	135	11.2	+94.0°
7	125	125	7.75	+94.1°

fication of the salt was carried out as seen from Table 5; $[\alpha]_D$ of the acid, liberated from small samples of each salt fraction, was determined in benzene.

The crude acid liberated from the last fraction was brownish red; yield 3.4 g. It was recrystallised four times alternately from 85 % formic acid and cyclohexane. 2.05 g of the pure (+)-acid were collected as small colourless needles. M.p. 100.8–101.8°.

91.48 mg acid: 8.21 ml 0.04823-*N* NaOH
$C_{14}H_{14}O_3$ calc. equiv. wt. 230.3
found » » 231.0

0.1827 g dissolved in abs. *alcohol* to 20.03 ml: $2\alpha_D^{25} = +0.120°$. $[\alpha]_D^{25} = +6.6°$; $[M]_D^{25} = +15.1°$.
0.1733 g dissolved in *acetone* to 20.03 ml: $2\alpha_D^{25} = +0.087°$. $[\alpha]_D^{25} = +5.0°$; $[M]_D^{25} = +11.6°$.
0.2141 g dissolved in *chloroform* to 20.03 ml: $2\alpha_D^{25} = +0.206°$. $[\alpha]_D^{25} = +9.6°$; $[M]_D^{25} = +22.2°$.
0.1042 g dissolved in *benzene* to 10.00 ml: $\alpha_D^{25} = +0.982°$. $[\alpha]_D^{25} = +94.2°$; $[M]_D^{25} = +217.0°$.
0.2200 g neutralised with *aqueous sodium hydroxide* and made up to 20.03 ml: $\alpha_D^{19} = +0.514°$. $[\alpha]_D^{19} = +46.8°$; $[M]_D^{19} = +107.8°$.

As none of the common alkaloids used for resolution gave a crystalline salt containing excess of the (−)-acid, an attempt was made to isolate the (−)-form by recrystallisation from different solvents of an acid containing about 85 % of the (−)-form but without success.

Optically active α-(2-naphthoxy)-n-butyric acid (III). A dextrorotatory acid could be isolated over the cinchonidine salt but the best result was obtained with quinine. Also α-phenylethylamine gave a crystalline salt and the (+)-amin salt was used for the preparation of the (−)-acid.

α-(2-Naphthoxy)-*n*-butyric acid (17.3 g, 0.075 mole) and quinine (28.4 g, 0.075 mole) were dissolved in hot 50 % ethanol (750 ml). After standing over night the crystals were collected and the rotatory power of the acid liberated from a small sample was measured in abs. alcohol. The salt was recrystallised several times as shown by Table 6.

Table 6. Recrystallisation of the quinine salt of α-(2-naphthoxy)-*n*-butyric acid.

Cryst. No.	50 % ethanol ml	Weight of salt (g)	$[\alpha]_D$ of the acid
1	750	29.8	+27.0°
2	700	19.5	+81.2°
3	500	16.4	+88.2°
4	400	15.5	+90.7°
5	350	14.8	+91.0°

The acid was isolated from the last salt fraction and recrystallised twice from petroleum (b.p. 60–80°). 5.5 g of the pure (+)-acid were collected as a colourless wad-like mass, m.p. 134.5–135.6°.

79.52 mg acid: 7.23 ml 0.04792-*N* NaOH
$C_{14}H_{14}O_3$ calc. equiv. wt. 230.3
found » » 229.5

0.1012 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{25} = +0.929°$. $[\alpha]_D^{25} = +91.8°$; $[M]_D^{25} = +211.4°$.
0.1006 g dissolved in *acetone* to 10.00 ml: $\alpha_D^{25} = +0.982°$. $[\alpha]_D^{25} = +97.6°$; $[M]_D^{25} = +224.8°$.
0.1006 g dissolved in *chloroform* to 10.00 ml: $\alpha_D^{25} = +0.620°$. $[\alpha]_D^{25} = +61.6°$; $[M]_D^{25} = +141.9°$.
0.1018 g dissolved in *benzene* to 10.00 ml: $\alpha_D^{25} = +0.856°$. $[\alpha]_D^{25} = +84.1°$; $[M]_D^{25} = +193.7°$.
0.1020 g neutralised with *aqueous sodium hydroxide* and made up to 10.00 ml: $\alpha_D^{25} = +0.686°$. $[\alpha]_D^{25} = +67.3°$; $[M]_D^{25} = +154.9°$.

From the mother liquors an acid with $[\alpha]_D = -55°$ (in ethanol) was regenerated. This acid (7.6 g, 0.033 mole) and an equimolecular quantity of (+)-α-phenylethylamine (4.0 g) were dissolved in hot alcohol (150 ml) and water (200 ml) was added. After some hours the crystals were collected and the optical activity of the acid determined in the usual manner. As seen from Table 7 only three recrystallisations were necessary to accomplish the resolution.

Table 7. Recrystallisation of the (+)-phenethylamine salt of α-(2-naphthoxy)-*n*-butyric acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Water	Ethanol		
1	200	150	8.3	−87.5°
2	135	100	7.4	−89.7°
3	135	100	6.4	−89.8°

From the (−)-acid salt 4.0 g of crude acid were obtained. It was recrystallised from petroleum (p.b. 60–80°), cyclohexane and 25 % alcohol. Yield of pure (−)-acid 3.7 g with m.p. 134.5–135.4°.

95.38 mg acid: 8.61 ml 0.04792-*N* NaOH
$C_{14}H_{14}O_3$ calc. equiv. wt. 230.3
found » » 231.2

0.1045 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{25} = -0.949°$. $[\alpha]_D^{25} = -90.8°$; $[M]_D^{25} = -209.1°$.

Melting point diagrams. The mixtures were prepared by dissolving weighed quantities of the components (a total of 10 mg) in a few drops of acetone; after evapora-

Table 8
(+)- and (−)-I.

% (+)-I	M.p.
0.0	79 –80°
10.3	71 –76°
15.4	71 –74°
20.0	71 –72.5°
24.5	75°
29.7	71 –78.5°
40.0	71 –81.5°
50.0	81.5–83°
100.0	79 –80°

Table 9
(+)- and (−)-II.

% (+)-II	M.p.
50.0	113.5–115°
60.0	94 –114°
70.2	94 –111.5°
79.7	94 –106.5°
85.1	94 –102°
89.2	94 – 96°
94.6	94.5– 99.5°
100.0	102 –103°

Table 10
(+)- and (−)-III.

% (+)-III	M.p.
0.0	134.5–135.5°
50.0	126 –127.5°
60.0	122 –126.5°
64.9	121.5–125°
69.6	122 –124°
75.0	122 –126°
79.7	123 –128.5°
89.1	124 –132.5°
100.0	134.5–135.5°

Table 11
(+)-α-Phenoxypropionic acid and (−)-I.

% (−)-I	M.p.
0.0	85 –86°
14.8	65 –78.5°
28.3	59 –71°
38.5	59 –64°
42.5	59 –61°
44.7	58.5–60°
48.3	59 –60.5°
50.0	61°
52.7	58 –60.5°
55.2	59 –60°
58.0	59 –61°
68.4	58.5–67.5°
84.7	59.5–75°
100.0	79 –80°

Table 12
(−)-α-Phenoxypropionic acid and (−)-I.

% (−)-I	M.p.
0.0	85 –86°
15.0	76 –79.5°
28.3	67.5–73°
33.2	64 –71°
41.4	61.5–67.5°
48.5	60.5–64.5°
52.5	61 –63°
58.4	61 –65°
68.7	64 –69°
82.8	71 –75°
100.0	79 –80°

Table 13
( )-α-(1-Naphthoxy)-propionic acid and (+)-II.

% (+)-II	M.p.
0.0	124 –126°
14.3	102 –120°
29.2	101 –112.5°
38.8	101 –106.5°
43.1	101 –103°
48.8	101.5–103°
58.5	91 –102°
67.9	90 – 99.5°
76.7	90 – 96.5°
83.4	90 – 93°
91.7	90 – 98°
100.0	101 –102°

Table 14
(+)-α-(1-Naphthoxy)-propionic acid and (+)-II.

% (+)-II	M.p.
0.0	123 –126°
14.5	107 –120.5°
30.2	98 –113.5°
37.9	94 –109°
48.4	91.5–102.5°
53.5	91 – 97.5°
58.3	91.5– 94°
60.4	91 – 93.5°
66.3	91.5– 94.5°
69.2	92.5– 95.5°
76.7	95 – 97.5°
84.2	97 – 99°
100.0	101 –102°

Table 15
(−)-α-(2-Naphthoxy)-propionic acid and (+)-III.

% (+)-III	M.p.
0.0	117.5–118.5°
5.8	109 –115°
10.9	108.5–112.5°
16.8	108.5–110°
28.2	109 –117°
39.6	109 –121°
49.6	120 –122°
58.9	118.5–121°
63.3	118.5–120°
70.2	118.5–123°
83.5	118.5–129.5°
100.0	134.5–135.5°

Table 16
(−)-α-(2-Naphthoxy)-propionic acid and (−)-III.

% (−)-III	M.p.
0.0	117.5–118.5°
5.4	111 –115.5°
8.3	109.5–114°
11.4	108 –112°
15.4	107.5–110°
20.8	108 –110.5°
24.9	108 –112.5°
29.6	109 –114°
39.1	111 –118°
48.4	115 –121°
58.5	118.5–124.5°
68.7	123 –127.5°
83.9	128.5–132°
100.0	135 –135.5°

tion of the solvent the residue was powdered and dried. Melting points were determined using a Kofler hot stage microscope (12). The results are to be found in Fig. 1–9 and Tables 8–16, where all compositions are given in mole-%.

The author is indebted to Prof A. Fredga for valuable discussions and for the supply of the optically active propionic acids used in this investigation. Thanks are due to Prof. N. Hellström, head of this institute, for his kind interest and for all facilities put at the author's disposal. The author is also indebted to Mr. S. Larsson for technical assistance with part of the synthetic work. Part of the expenses involved have been defrayed by a grant from *Statens Naturvetenskapliga Forskningsråd*, which is gratefully acknowledged.

SUMMARY

α-Phenoxy-*n*-butyric acid and the two isomeric α-naphthoxy-*n*-butyric acids have been resolved into optical antipodes. The racemic forms have been shown to be true racemates. The optically active forms have been sterically connected to the homologous propionic acids by the method of quasi-racemates. The results obtained hereby were found to be in agreement with WINTHER's rule and optical activity measurements. The acids which have greater auxin activity than their antipodes were found to belong to the same steric series as D-glyceric aldehyde.

Department of Organic Chemistry, Royal Agricultural College, Uppsala, February 1953.

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The valence of vanadium in hemolysates of bloodcells from *Ascidia obliqua* Alder

By Sigvar Lybing

With 6 figures in the text

Since Henze (1) discovered the presence of vanadium in bloodcells of *Phallusia mammillata* (Cuv.), several papers on the occurrence of vanadium in other species have been published (for references see Webb (2) and Bertrand (3)). From these works it is clear that vanadium is accumulated in greater amounts chiefly within the family *Ascidiidae*. There has been some speculation over the importance of vanadium to the organism, but so far it has been impossible to ascribe to it any physiological role whatsoever.

According to Henze the vanadium is bound to an organic compound. The complex is coloured and has been referred to as a chromogen. As possible functions of the vanadium chromogen the following have been set forth: 1) respiratory pigment, 2) metabolic carrier and 3) reduction system in the synthesis of tunicin (cellulose) from carbon dioxide.

A function as oxygen transporter has been excluded by several workers: Winterstein (4), Henze (5), Webb (2) and Califano and Boeri (6).

Some facts indicating a role as metabolic carrier are: The chromogen was found by Henze (5) to be a strong reducing agent and was suggested by him to act as a hydrogen donator being reversibly oxidized and reduced. This hypothesis was later (7) abandoned, as he considered the oxidation-reduction potential at which the chromogen would be reversibly oxidized and reduced to be so low as probably to lie outside the physiological range. Boeri (8), who measured the oxidation-reduction potential in hemolysates of bloodcells from *Phallusia mammillata*, gives a pH-potential curve, which shows a E_0'-value of 0.330 volt at $\mathrm{pH} = 0.5$ decreasing with increasing pH to 0.085 volt at $\mathrm{pH} = 3$. The exact pH-value inside the vanadium-containing cells, called vanadocytes by Webb (2), is not known, but as these cells, according to Henze (5), contain free sulfuric acid in high concentration, very low pH-values of the medium surrounding the chromogen must be considered as physiological. Thus, contrary to what was assumed by Henze (7) the potential is rather high and the chromogen should then easily be reduced again when once oxidized. It must however be remembered that Boeri (8) measured the potential of the chromogen in hemolysates, and as it is not known what changes the chromogen may undergo on hemolysis, nothing final can be said about its oxidation-reduction potential *in vivo*.

Regarding a function in the tunicin-synthesis, HENZE (7) put forward the possibility of the vanadium chromogen as a reduction-system similar to that of the chloroplasts, possibly by using light energy, which would mean an uptake of carbon dioxide under reduction and synthesis of carbohydrate. The assumptions on which this hypothesis were based have, however, turned out to be false. The chromogen is evidently not a strong reducing agent and the low CO_2-content of the plasma, reported by WINTERSTEIN (4) and HENZE (7), is not due to a continuous withdrawal of CO_2, but is ascribed to the low alkaline reserve of the plasma (FLORKIN (9)).

An assumption of the chromogen as an intermediary metabolic carrier seems to be the most promising approach in elucidating its physiological role. As very little is known about the chemical constitution and the physico-chemical properties of the vanadium compound, it is of importance to get more information about these problems before attempting any thorough physiological investigation.

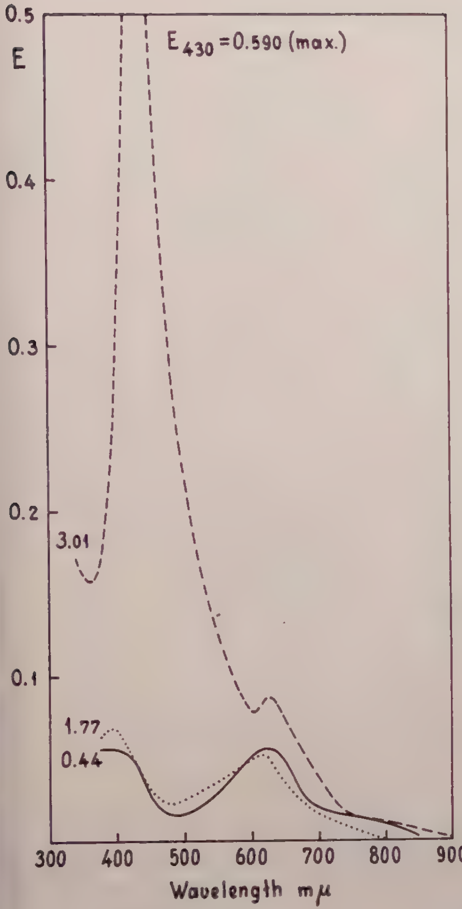
The valence of vanadium and a possible valence change is of great interest in connection with a presumed function as an intermediary carrier. Further, as the conclusions, drawn by HENZE (7) and CALIFANO and BOERI (6) on the presence of vanadium as bivalent in the chromogen, cannot be regarded as valid (see below), this investigation was undertaken in order, with spectrophotometric methods, to try to establish the valence of the vanadium. In the paper by BOERI (8), which came to the author's notice after the completion of this investigation, stress is laid upon the fact that the sole basis for the assumption of the vanadium as bivalent is the ability of both a hemolysate and a bivalent vanadium solution to reduce methylene blue and indigo carmine.

Material and methods

Ascidia obliqua ALDER was chosen for this investigation because of its high vanadium content and the ease with which it could be supplied in sufficient amounts. The animals were freed from the tunic and the blood was obtained by cutting through the heart. By centrifuging the blood at about 2000 r.p.m. for 2 min. the cells were distributed in two distinct layers, an upper layer containing the large, vesicular, uncoloured cells and a bottom layer of different types of mainly green cells. 10 µl of these green cells contain about 0.1 mg vanadium, and the extinction measurements were made on hemolysates of these bottom layer cells, with a Hilger photoelectric spectrophotometer (UVISPEK). The hemolysates, after filtration through ordinary filter paper to get clear solutions, were measured in 1 cm cells except when absorption curves were taken on evacuated hemolysates when Thunberg tubes were used. (The fact that these were not exactly isodiametric accounts for the different extinction values obtained at longer wavelengths (Fig. 1). No difference was to be expected as equal volumes of the same hemolysate were acidified, after evacuation, with equal amounts of sulfuric acid of various pH.) For the vanadium determinations the method of WRIGHT and MELLON (10) was followed. The organic material was removed by wet digestion with about 3 parts of 70 % perchloric acid and 1 part of concentrated sulfuric acid. The amount of sulfuric acid added was chosen so to get a final concentration, after dilution and addition of the reagents, of about 0.5 N H_2SO_4. The extinction was measured with the Hilger UVISPEK at 400 mµ in 1 cm cells.

Results

The absorption curves (Fig. 1), measured on hemolysates of different pH-values (hemolysis in distilled water and thereafter acidification with H_2SO_4 of different molarities), show for $pH < 2$ two maxima at about 400 mµ and 625 mµ. At $pH > 2$



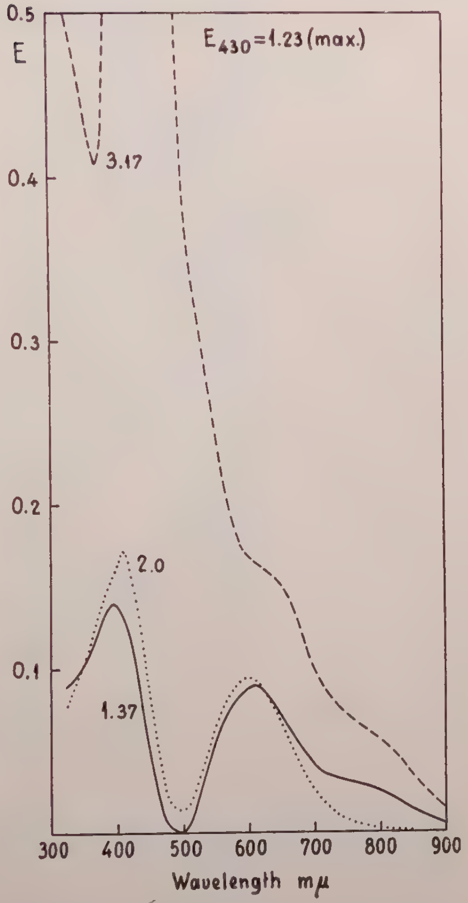


Fig. 1. Absorption spectra of *Ascidia obliqua* hemolysates of various pH-values. (The hemolysates evacuated 30 min. after hemolysis, acidified and measured in Thunberg tubes.)

Fig. 2. Absorption spectra of a trivalent vanadium solution at various pH-values. (The solutions of pH 1.37 and 3.17 are slightly oxidized.)

a new band with a maximum at about 430 mμ begins to appear and is getting stronger when the pH of the hemolysate is increased. This band is thus dependent on the pH. The hemolysates are, on standing in the air, oxidized with different rates to a uniform final state. The absorption maximum for these oxidized hemolysates lies at 760 mμ and the curves correspond entirely with the absorption curve for a solution of vanadyl sulfate ($VOSO_4$) in distilled water. The ion VO^{2+} (tetravalent vanadium) is thus responsible for this maximum, see also FURMAN and GARNER (11). The maxima at 400 mμ and 625 mμ of an unoxidized hemolysate can be ascribed to the hydrated ion V^{3+} and the maximum at 430 mμ is due to the ion VOH^{2+} (trivalent vanadium), formed by hydrolysis of V^{3+} according to the reaction: $V^{3+} + H_2O \rightleftharpoons VOH^{2+} + H^+$. This conclusion is drawn from a comparison with the absorption curves obtained by FURMAN and GARNER (11) on $V(ClO_4)_3$-solutions in perchloric

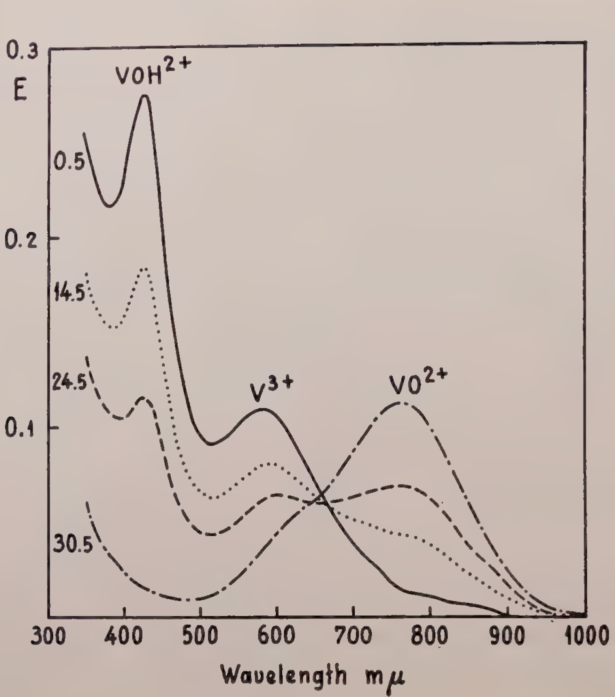


Fig. 3. Absorption spectra of an autoxidizing *Ascidia obliqua* hemolysate in glycine-HCl-buffer of pH 3.0, measured at different times (hours) after hemolysis.

acid with maxima at 400 mμ and 580 mμ, accounted for by the absorption of the hydrated ion V^{3+}. It was further shown by them that the extinction increased as the acidity of the solution was decreased. The greatest increase occurred at 430 mμ and was proved to be due to the formation of the ion VOH^{2+}.

This interpretation of the bands points to the fact that the vanadium to a great extent is present in the hemolysates as free inorganic ions and by reducing a vanadyl sulfate solution one could expect to obtain a solution, whose absorption spectrum should correspond with those of hemolysates. This is also shown to be the case (Fig. 2). These curves are measured on a solution obtained from a vanadyl sulfate solution by reduction with zinc and brought to different pH-values by addition of H_2SO_4 or KOH. The divergent extinction values at longer wavelengths ($>$ 700 mμ) depend upon the oxidation of the original solution of pH 2.0.

Hemolysates in different buffer solutions give similar absorption curves. A hemolysate in a glycine-HCl-buffer of pH 3.0 may be given as an example (Fig. 3). These curves, measured at different times after hemolysis, also describe graphically the changes taking place when a hemolysate is oxidized. Here three well-defined bands can be seen at 428 mμ, 585 mμ and 760 mμ, each of which depends upon the presence of the ions VOH^{2+}, V^{3+} and VO^{2+} respectively. The isosbestic point at about 650 mμ indicates that there is a transition from one resonating system to another. The apparent shift of the 585 mμ band to longer wavelengths is an additional effect of the decreasing 585 mμ band and the increasing 760 mμ band.

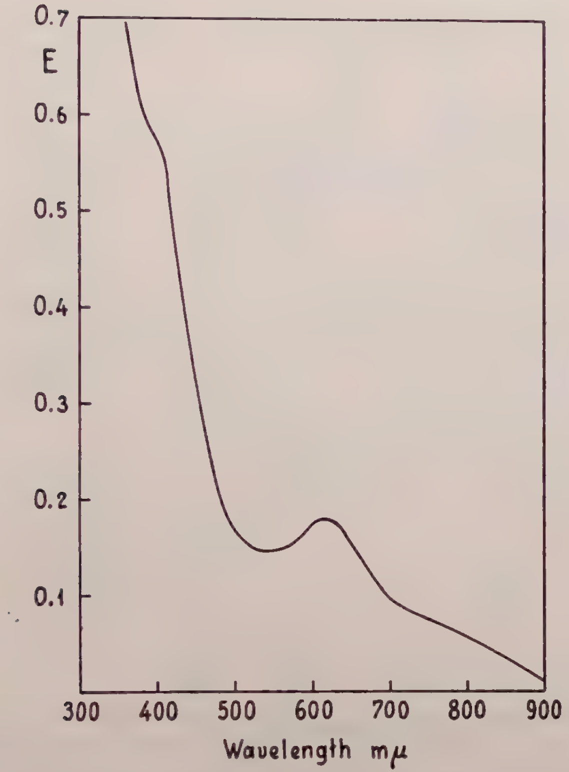


Fig. 4. Absorption spectrum of dried *Ascidia obliqua* bloodcells.

From these spectrophotometric experiments it is clear that vanadium in hemolysates of bloodcells from *Ascidia obliqua* is present as trivalent and on oxidation changes to tetravalent.

Now the question arises whether the vanadium occurs as trivalent also *in vivo.* In the capillaries permeating the branchial walls the green coloured bloodcells are very numerous, and by letting animals dry in the air, after removal from the tunic, this branchial region forms a thin, strongly green coloured membrane. The absorption spectra, measured on this membrane, have a maximum at 615 mμ and show a deflection at about 400 mμ (Fig. 4), which points to the presence of trivalent inorganic vanadium in the dried cells. By hemolysis of acetone dried cells, solutions are obtained, the absorption curves of which correspond with those of hemolysates of living cells. This indicates that drying and hemolysis have the same effect upon a possible organic vanadium compound present in living cells. It is hoped that, if such an organic vanadium compound exists, its presence may be revealed by measuring the absorption spectra on living bloodcells.

Discussion

By assuming a physiological role of the vanadium as an intermediary carrier, functioning as inorganic ion or combined with some organic compound, addition of Na-dithionite to a hemolysate would be expected to cause a shift of the absorption

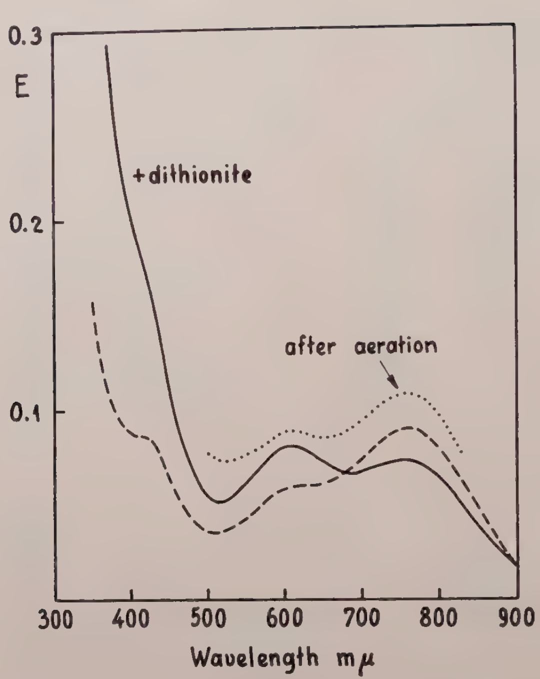


Fig. 5. Changes in the absorption spectrum of a partly oxidized *Ascidia obliqua* hemolysate (— — —) on addition of Na-dithionite (———) and on subsequent aeration (····); pH 3.0, glycine-HCl-buffer.

bands or at least a change in the relative band intensities, a change being reversed on bubbling air through the solution. When a partly oxidized hemolysate of pH 3.0 in a glycine-HCl-buffer is reduced with dithionite, a change in the relative band intensities is obtained, revealing a reduction of VO^{2+} to V^{3+}. By bubbling air through the solution, some of the trivalent vanadium formed is oxidized back to tetravalent (Fig. 5). Whether this oxidation-reduction reaction takes place in the living blood-cells or not is an open question, but the experiment indicates that such a function is possible.

The curve in Fig. 5 representing the dithionite-reduced solution, shows no maximum at 430 mμ although the pH-value is as high as 3.0. This 430 mμ band was never obtained at any of the pH-values used, when an oxidized hemolysate or a vanadyl sulfate solution were reduced with dithionite. A yellow hemolysate with an absorption maximum at 430 mμ is further decolourized on addition of dithionite. It seems probable that the ion VOH^{2+} is not stable in dithionite-reduced solutions.

One might however suspect that, when using very small amounts of dithionite, some VOH^{2+} is still present in the solution masked by the absorption of the reaction products formed. This is based upon the assumption that the oxidation of trivalent vanadium goes over the hydrolyzed ion VOH^{2+}. In some dithionite-reduced hemolysates no back-oxidation to tetravalent vanadium occurred upon aeration.

In a work by RAMSEY, SUGIMOTO and DEVORKIN (12) on the kinetics of the oxidation of trivalent vanadium it is considered as significant that "non-oxide"

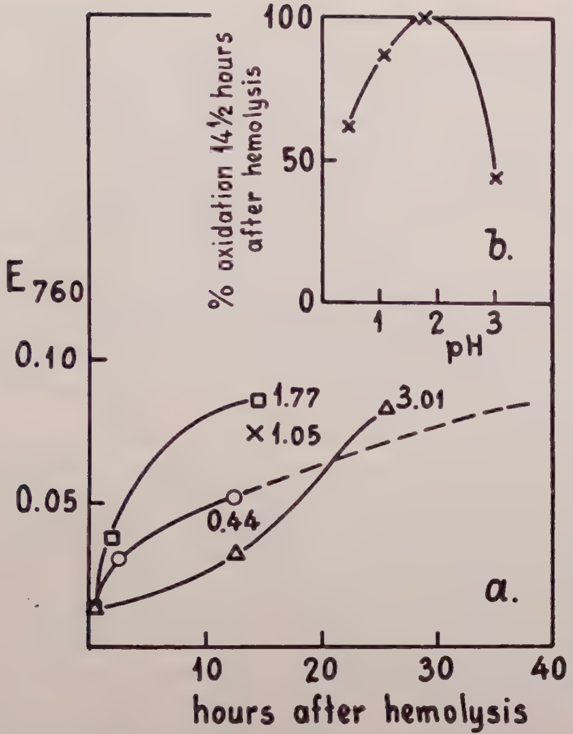


Fig. 6. a) Rates of oxidation of *Ascidia obliqua* hemolysates of various pH-values (equal vanadium conc.). b) Relative rates of oxidation in relation to pH. (For further explanations see text.)

containing oxidants as oxygen and bivalent copper react with measurable rates only with hydrolyzed trivalent vanadium (VOH^{2+}) even though this ion constitutes but a very small part of the total trivalent vanadium present in the acid solutions used.

If the oxidation of hemolysates of various pH-values is followed by measuring the absorption at different times after hemolysis, it is found that they are oxidized at different rates (see Fig. 6 a where the extinction at 760 mμ (conc. of VO^{2+}) is recorded against the time after hemolysis). The total vanadium concentrations of the different solutions were equal, and by assuming the final concentrations of VO^{2+} also to be the same (this is probably not quite true owing to the hydrolysis of VO^{2+} at higher pH), the concentration of VO^{2+} at a certain arbitrary time will be a measure of the relative velocities with which the oxidation occurs in the various solutions used. In fig. 6 b the concentrations of VO^{2+}, 14.5 hours after hemolysis, given in per cent of the final VO^{2+}-concentrations, are represented as a function of the pH-values of the hemolysates. The curve shows that the oxidation takes place with maximal rate at pH-values of about 2.

The formula $-\frac{d(V^{3+})}{dt} = k\frac{(O_2)(V^{3+})}{(H^+)}$ ((V^{3+}) standing for the total conc. of trivalent vanadium) given by Ramsey and co-workers (12) for the velocity of the oxidation of trivalent vanadium, discloses that the rate is inversely proportional to the hydrogen ion concentration. An increase in the oxidation rate with decreasing acidity is thus to be expected.

The diverging results obtained with hemolysates of different pH suggest, at a first thought, the presence of some pH-dependent factor controlling the oxidation rate. Ramsey *et al.* (12) worked however with solutions of pH-values below 2, and at higher pH-values complications set in due to the further hydrolysis of the ions VOH^{2+} and VO^{2+}. In the paper by Furman and Garner (11) 2×10^{-3} is given as an approximate value for the first hydrolysis constant ($V^{3+} + H_2O \rightleftharpoons VOH^{2+} + H^+$). An approximate value of the constant for the second step: $VOH^{2+} \rightleftharpoons VO^+ + H^+$ can be calculated from the following data given by Lingane and Meites (13). From polarographic measurements in an acetate-buffer of pH 5.4, these workers found the ratio VO^+/VOH^{2+} to be $\geqq 1.44$. Thus 5.7×10^{-6} is obtained as a minimum value for the second step. These constants are of the same order of magnitude as those determined for Fe^{3+} by Lamb and Jacques (14).

A thorough quantitative interpretation cannot be based on these approximate constants, but it seems not improbable that the pH-optimum for the oxidation rate can be explained by the complex reactions taking place in an inorganic trivalent vanadium solution.

The pH-value of about 2.2 found by Boeri (8) for mechanically ruptured cells of *Phallusia mammillata* does perhaps not represent the true inner acidity of the cells, but is of interest in connection with the pH-optimum of about 2, found for the rate of the oxidation of tri- to tetravalent vanadium. Whether this conformity is of any physiological significance may be revealed by further experiments.

A comparison between the absorption curves measured by Boeri (8) on hemolysates of bloodcells from *Phallusia mammillata* and those obtained in this investigation shows that the vanadium must be rather firmly bound to some compound in the case of *Phallusia*, as no free inorganic vanadium seems to appear in the hemolysate as least at low pH-values. It is probable that the small maximum at 420 mμ in Boeri's curves at higher pH can be ascribed to the ion VOH^{2+}. That some vanadium is set free was shown by Califano and Boeri (6) who found vanadium in the outer solution on dialysis at pH 2.4.

The absorption spectrum on dried *Ascidia obliqua* cells (Fig. 4) indicates the presence of trivalent inorganic vanadium (absorption band at 615 mμ), but this must not be taken as evidence for its occurrence in inorganic form in the living cells. Both drying and hemolysis of *Ascidia obliqua* cells may cause the release of inorganic vanadium from an organic complex, even though this is apparently not accompanied by any change in valence.

Conclusions regarding the valence state in which vanadium is bound in the *Phallusia*-chromogen cannot be drawn from the absorption curves presented by Boeri (8), but the high E_0'-value for the chromogen and the shape of his oxidative titration curve suggest rather a trivalent vanadium complex than the bivalent one proposed. This suggestion is based on the following interpretation of Boeri's titration curve on the *Phallusia*-chromogen. The first two univalent steps correspond to the oxidation of V^{3+} over V^{4+} to V^{5+}, and the latter part of the curve represents the bivalent oxidation of some compound present in the solution; the two one-electrone steps not separated owing to the low semiquinone formation constant of the compound.

SUMMARY

Some aspects of interest in connection with a presumed physiological function of vanadium in ascidians are briefly summarized.

The valence of vanadium in hemolysates of *Ascidia obliqua* bloodcells is established.

The autoxidation of hemolysates is followed spectrophotometrically and the oxidative course is discussed.

Observations on *Ascidia obliqua* hemolysates are compared and discussed in relation to earlier results reported on the vanadium chromogen of *Phallusia mammillata*.

This work has been carried out during the summer of 1952 at the Kristineberg Zoological Station of the Royal Swedish Academy of Science and the author wishes to express his gratitude to the Station Staff for their generous help in supplying the material. I am also much indebted to Professor Hans G. Borei for his encouragement and kind interest in the work.

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Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 22

Communicated 13 May 1953 by SVEN HÖRSTADIUS and PER ERIC LINDAHL

Paper chromatographic separation of thiamine and its phosphate esters

By KARL-HEINZ KIESSLING and GUNBORG LINDAHL

With 1 figure in the text

In a previous work on cocarboxylase as a phosphate carrier (1) a paper chromatographic separation method was used for the final purification of cocarboxylase (thiamine-diphosphate) from other phosphate compounds. This method was earlier used for the separation of adenosine polyphosphates by COHN and CARTER (2) and, after modification, by SNELLMAN and GELOTTE (3). For our present purpose, however, this separation method is not satisfactory because of its incapacity of separating thiamine-diphosphate (TDP) from thiamine-triphosphate (TTP). ROSSI-FANELLI *et al.* published a paper chromatographic separation mehtod in connection with a TTP-analysis on the liver (4), but also here the separation of TTP and TDP was not satisfying..

We are at present investigating the occurrence of TTP and TDP in different tissues and cell-fragments, the mechanism behind the appearance of these two compounds and their role in the intermediary metabolism. For this reason we have worked out a paper partition method separating the two compounds from each other better than earlier methods do, and keeping the spots small and concentrated. By testing several different types of solvents, alcohols were found to have the best capacity of separating TTP and TDP. *n*-bytulic alcohol appeared most suitable, especially when mixed with formic acid. Thus *n*-butylic alcohol, saturated with water, and containing 18 % formic acid, was used. In experiments in which the synthetic esters were chromatographed[1] with this solvent, chromatograms similar to that in Fig. 1 appear.

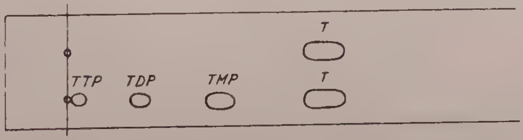

Fig. 1. The mutual situation of thiamine (T), thiamine-monophosphate (TMP), thiamine-diphosphate (TDP) and thiamine-triphosphate (TTP), when chromatographed for 16 hours with the descending technique. Paper: Whatman 1, washed with 4 N HCl and oxine according to HANES and ISHERWOOD (5). The spots are made visible by spraying the paper with $K_3Fe(CN)_6$ in an alkaline solution.

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[1] We are very much indebted to Professor L. VELLUZ for the gift of a sample of TTP.

This method may be used also for separating thiamine-phosphates in biological materials. This was shown by chromatographing protein-free liver extracts to which thiamine-phosphates had been added. The separation was nearly as good as with synthetic phosphate compounds alone.

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Read 22 April 1953

A new method for the preparation of secretin

By Erik Jorpes and Viktor Mutt

With the assistance of Per Wallén

Since the discovery of secretin in 1902 by Bayliss and Starling numerous methods have been applied for its extraction and purification. They have been extensively reviewed by Greengard (1). Extraction of the intestine with dilute aqueous hydrochloric acid, followed by precipitation of the extract with sodium chloride occurred in most of the procedures. The sodium chloride precipitate, originally introduced by Weaver, Luckhardt, and Koch 1926 (2), has been a convenient starting material for the further purification of secretin (3).

Following up the work of Dale and Laidlaw (4, 5), Ågren (6) and Hammarsten (7), to whom we are indebted for much of our knowledge concerning the chemical properties of secretin, used a dilute sulphuric acid extract of the intestine from which the secretin was precipitated either with mercuric sulphate or with sodium sulphate.

Aqueous extracts are, however, difficult to handle, especially when working on a large scale. Mellanby (8), at an early date, tried to avoid this handicap by extracting the intestinal mucosa with ethanol. The purification of the crude extract was, however, rather complicated and did not yield a pure product.

Other workers too have experienced difficulties with the purification of the crude secretin. A variety of methods have been worked out for this purpose. Thus fractionating with organic solvents, precipitation from aqueous solution with picric, picrolonic or trichloracetic acid, electrodialysis and many other procedures have been tried with varying success. Secretin being a basic substance as demonstrated by Hammarsten och Ågren (6, 7) it occurred to us that it should be amenable to purification through ion-exchange. Experimental work soon verified this assumption.

In the past few years the technique of ion exchange has been applied to the purification of various biologically active substances, even of high molecular weight. Astwood, Raben, Payne and Grady (9) used oxycel for the purification of the adrenocorticotrophic hormone. Amberlite IRC 50 was used by Dixon et al. (10) for the same purpose.

We found that secretin is obtained in good yield by extracting the intestine with ethanol acidified with acetic acid, adsorbing the active principle on a carboxylic cat-ion exchanger, eluting with hydrochloric acid in ethanol, and precipitating the active material from the eluate with cold acetone.

The crude material, thus obtained, could be purified by isoelectric removal of impurities from a methanolic solution as described by Jorpes and Mutt (11, 12). Water-soluble impurities of low molecular weight including histamine are removed by precipitating the purified product with sodium chloride at saturation.

Of the various types of ion-exchangers tried by us (amberlite IRC-50; alginic acid; oxycel; pectic acid) we found citrus pectic acid (polygalacturonic acid) to be the most satisfactory. Incidentally lemon peel, minced, and washed in turn with water, ethanol, 0.1 N sodium hydroxide in ethanol, 0.1 N hydrochloric acid in ethanol, and ethanol, worked fairly well in several instances. Some batches of the peel, however, were less satisfactory. We therefore preferred to use the citrus pectic acid. The following method gives, if properly adhered to, secretin in lyophilized form, with an activity of 1200 cat units per mg ash free substance, i.e. 0.01 mg causes on the average a secretion of 1.2 ml of 0.1 N bicarbonate solution.

Experimental

Pretreatment of the intestines.

The uppermost 1 m of the intestine was removed from the hogs not later than half an hour after slaughter, emptied of its contents and immersed in ice water. After cooling for half an hour the pieces of intestine were everted, and the mucus covering them washed away manually using ice water. The material was then squeezed dry and frozen at −20° C. Storage at this temperature for several months did not affect the yield, provided that drying was prevented by storage in closed containers.

Preparation of the crude secretin.

10 kg of the frozen material (from about 100 hogs) were extracted for 12 hours at +5° C by vigorous shaking in glass flasks with 20 l of 95% ethanol containing 300 ml of glacial acetic acid. The intestines were then squeezed dry. They contained considerable quantities of pancreozymin and cholecystokinin but only little secretin.

The extract was filtered by suction with the aid of 500 g of Hyflo super cel. The clear filtrate was allowed to stand at −20° C for 24 hours. The precipitate formed during this time was filtered off and discarded. The filtrate was placed in a room at +4° C and treated with the pectic acid.[1]

Prior to use it was necessary to wash the pectic acid with water so as to remove water-soluble depolymerization products of low molecular weight. This was accomplished by suspending 500 g of the pectic acid in 10 l of cold distilled water. After allowing the insoluble matter to settle the supernatant was discarded. This procedure was repeated until the supernatant no longer reacted acid to congo red. The insoluble residue was then washed with 80% ethanol and collected on a suction filter. It was mixed with the extract from the intestines and the mixture stirred for 18 hours at +4° C. After this time the pectic acid, containing the adsorbed secretin, was allowed to settle for a few hours. It was then collected on a suction filter and washed three times with 500 ml volumes of 80% ethanol. It was not pressed dry, but allowed to remain rather moist during these washings. For elution the material was transferred to a beaker and stirred at +4° C for 15 min. with 2000 ml of 0.2 N HCl in 80% ethanol. The insoluble material was then filtered off and resuspended in 2000 ml of 0.1 N HCl in 80% ethanol. After 15 min. stirring it was filtered off. The combined filtrates were precipitated with four volumes of cold (−20° C) acetone. The acetone precipitate was collected and washed on a suction filter five times with acetone and thrice with ethyl ether. The ether was allowed to evaporate. The weight of the crude material varied somewhat in different preparations. The average yield of ten preparations was 3.44 g (1.5; 1.7; 5.8; 6.7; 2.3; 2.6; 4.0; 4.6; 1.9; 3.3). The secretin activity was about 100 cat units per mg, except in the preparation with the yield of 1.7 g, where it was about 200 c.u./mg, and in the preparation with the yield of 5.18 g, where it was about 150 units.

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[1] Obtained from the California Fruit Growers Exchange, Ontario, California.

As could be expected the material was contaminated with vasodilator substances. The pectic acid was washed with water and 80% ethanol and was ready for renewed use.

Purification of the acetone precipitate.

10 g of the acetone precipitate were dissolved in 200 ml of distilled water at 0° C. 1800 ml of methanol (0° C) were added and the pH of the solution brought to 6.5–7. with 0.2 NaOH in 90% methanol. This and all following measurements of pH were made with a glass electrode. The precipitate was filtered off and discarded. The pH of the filtrate was brought to 3.0 with 1 N HCl in 90% methanol. Three volumes of cold (−20° C) acetone were added and the precipitate was collected on a suction filter. After washing on the filter with acetone and ether it was dissolved in 50 ml of cold distilled water (+5° C) and then salted out with 16 g of sodium chloride. The sodium chloride precipitate was collected on a suction filter, washed on the filter with a cold, saturated solution of sodium chloride, and pressed dry. It was then dissolved in 50 ml of cold distilled water and reprecipitated with 16 g of sodium chloride. The reprecipitated material was collected on a suction filter, washed with the saturated sodium chloride solution and pressed dry. It was then dissolved in distilled water, and the solution lyophilized. The lyophilized material weighed about 2.0 g, contained about 20% ash and had a secretin activity of about 300 c.u. per mg. It was free of vasodilator substances.

Further purification.

2 g of the lyophilized powder were dissolved in 40 ml of cold distilled water. 760 ml of cold methanol were added and the pH of the solution brought to 6.5–7.0 with 0.1 N NaOH in methanol. After 24 hours at −20° C the precipitate was filtered off and discarded. The filtrate was precipitated with four volumes of ethyl ether. The precipitate was collected in the cold room on a suction filter, washed twice with ether and then dissolved in distilled water. The solution was lyophilized. The lyophilized material weighed about 0.3 g, contained 50% ash and had an activity of 1200 c.u. per mg ash-free substance.

Summary

A new method has been worked out for the preparation of highly active secretin.

1) Crude secretin was prepared by extracting the upper first meter of the hog small intestine with ethanol acidified with acetic acid, adsorbing the active material on pectic acid, eluting with hydrochloric acid in ethanol, and precipitating with acetone. The average yield of ten preparations was 3900 cat units per meter of intestine. The samples had an activity of at least 100 units per mg. A yield of 6000–8700 units per meter was occasionally obtained.

2) Purification was achieved by isoelectric removal at pH 7 of impurities from a solution of the crude material in 90% methanol at +4° C. The active material was

precipitated from the methanolic solution with acetone and further purified by repeated salting out with sodium chloride from aqueous solution. After lyophilyzing the material had an activity of 300 units per mg. The yield at this stage was 2000 units per meter.

3) For further purification impurities were removed through isoelectric precipitation at pH 7 from 95% methanol at − 20° C. The active material was then precipitated with ether and lyophilized. The activity of the lyophilized product was very high, 1200 c.u. per mg ash-free substance. The yield was, however, rather poor, i.e. about 600 units per meter of intestine.

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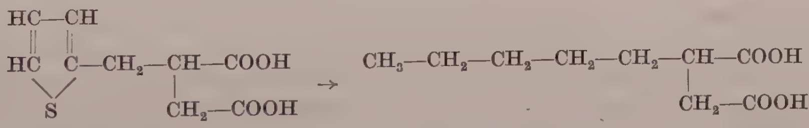
Optically active thiophene compounds. IV. On the use of thiophene derivatives for steric correlation of aromatic and aliphatic compounds

By Arne Fredga

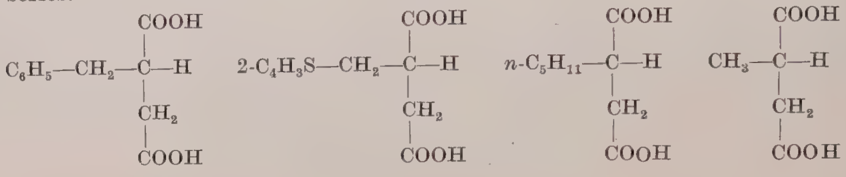
It is often difficult to connect aromatic compounds to the steric system of the glyceraldehyde (glycerose). Owing to its special electronic structure, an aromatic group near the asymmetric center exerts a dominating influence on the rotatory power. A comparison of activity data for aromatic and aliphatic compounds is therefore in most cases of little or no value. By hydrogenation of the benzene ring, more favourable conditions are obtained (1), but even in this case the influence of the cyclic stucture may be difficult to estimate. A study of phase diagrams cannot in these cases be expected to give any definite information, and direct conversion of benzene derivatives into acyclic compounds with retention of the optical activity is often out of question.

The steric relationships between analogous derivatives of benzene and thiophene can generally be established in different ways, for instance by phase studies. On the other hand, thiophene compounds can be converted into sulphurfree acyclic systems by treatment with Raney nickel (2). As it seems, this reaction has not previously been applied to optically active systems. In the cases studied by the present author no racemisation takes place, and the aromatic compounds are thus directly connected to aliphatic compounds. For most purposes, the 2-thienyl derivatives, being more readily accessible and giving *n*-butyl derivatives, are preferable. 3-Thienyl derivatives give derivatives of *sec*-butyl containing a new asymmetric center. This means of course in most cases a complication but might under special conditions be utilised for configuration studies.

The present paper deals with the formation of *n*-pentylsuccinic acid from 2-thenylsuccinic (α-thenylsuccinic) acid:



It was found that the acids with the *same* configuration have also the *same* direction of rotation. As earlier reported (3), the 2-thenylsuccinic acid can be connected to the benzylsuccinic acid by formation of a quasi-racemic compound. The *n*-pentylsuccinic acid has also been synthesised by conventional methods and resolved into the antipodes with strychnine and brucine. Using the author's preparations, MATELL has shown (4) that the active n-alkylsuccinic acids (up to n-hexyl) with the same direction of rotation have also the same configuration. As the methylsuccinic acid has been connected to the system of the glyceraldehyde (5) we get the following steric series:



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{C_6H_5{-}CH_2{-}C{-}H} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array} \qquad \begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{2\text{-}C_4H_3S{-}CH_2{-}C{-}H} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array} \qquad \begin{array}{c} \mathrm{COOH} \\ | \\ n\text{-}\mathrm{C_5H_{11}{-}C{-}H} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array} \qquad \begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{CH_3{-}C{-}H} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array}$$

(—) (—) (—) (—)

Preliminary experiments have shown that 2-thienylsuccinic acid can be converted into n-butylsuccinic acid and 2-(2-thienyl)ethylsuccinic acid into n-hexylsuccinic acid. Experiments with thienylsubstituted hydroxy and amino acids are in progress.

Experimental

rac.-n-Pentylsuccinic acid. The acid was prepared by alkylation of ethyl ethane-1.2.2-tricarboxylate and subsequent hydrolysis and decarboxylation. The reaction was performed in the usual way in ethanolic solution using 5.8 g (0.25 mole) of sodium, 61.5 g (0.25 mole) of ethyl ethane-1.2.2-tricarboxylate and 45 g (0.30 mole) of n-pentyl bromide. The yield of ethyl heptane-1.2.2-tricarboxylate, boiling at 170°–175° (8 mm Hg) was 63 g (80 %). The ester was refluxed with 400 ml 20 % hydrochloric acid until a homogeneous solution was obtained, the hydrochloric acid was distilled off and the crystalline residue (33 g) recrystallised several times from hydrochloric acid. The acid is very soluble in water and in most organic solvents, sparingly so in strong hydrochloric acid. The recrystallisation requires some care and attention as the acid has a tendency to separate as an oil. The most convenient mode of operation is to add concentrated hydrochloric acid with stirring to a not too concentrated aqueous solution of the acid at room temperature until the solution becomes slightly turbid. The acid will then separate in good yield on standing. It forms microscopic needles with m.p. 85–87°.

0.1602 g acid: 15.91 ml 0.1069–N NaOH. — 17.37 mg acid: 36.48 mg CO_2 and 13.07 mg H_2O.

		Equiv. wt.	C	H
$C_9H_{16}O_4$ (188.2)	calc.	94.1	C 57.43	H 8.57
	found	94.2	57.31	8.42

The melting point shows some irregularities probably due to polymorphism. MATELL (4) has reported an unstable modification, melting at c:a 72°, and it seems possible that more modifications exist. The racemic methylsuccinic acid shows a similar behaviour (6).

Resolution of the racemic acid. In preliminary experiments, strychnine and brucine were found to give the best results.

22.6 g (0.12 mole) of *rac.-n*-pentylsuccinic acid were dissolved in 450 ml of 50 % ethanol. The solution was heated to boiling, saturated with strychnine (c:a 75 g), filtered and placed in a refrigerator. After 24 hours, the salt was filtered off and recrystallised several times from dilute ethanol. On dissolving the salt, a small amount of strychnine separated owing to hydrolysis; it was brought into solution by adding a few drops of acetic acid. The progress of the resolution was tested on small samples from each crystallisation, from which the acid was liberated and examined in ethanolic solution.

Crystallisation	1	2	3	4
ml water	225	350	150	130
ml ethanol	225	150	70	60
g salt obtained	45.7	34.3	30.6	27.4
$[\alpha]_D$ of the acid	+ 21°	+ 26°	+ 26.5°	+ 26.5°

The mother liquor from the first crystallisation was evaporated to dryness and the residue recrystallised from the successive mother liquors obtained in the first series of crystallisations. The resulting salt was recrystallised from 60 ml water + 40 ml ethanol, yielding 15.6 g salt of pure (+)-acid. The total yield of strychnine salt was thus 42.0 g.

The mother liquors from the two first crystallisations of the second series were combined and the acid was liberated; 10.5 g having $[\alpha]_D = -22.5°$ were obtained. This acid (0.055 mole) was dissolved with 44.0 g (0.11 mole) of anhydrous brucine in 150 ml of water and the salt was recrystallised to maximum activity.

Crystallisation	1	2	3	4
ml water	150	100	100	100
g salt obtained	51.5	48.0	44.2	41.8
$[\alpha]_D$ of the acid	− 24°	− 25.5°	− 26°	− 26°

(+)-n-Pentylsuccinic acid. The strychnine salt (27.4 g) was decomposed with sodium carbonate solution and the strychnine extracted with chloroform. The acid was isolated from the sodium salt solution by acidification with sulphuric acid and extraction with ether. It was recrystallised from a little water and twice from ligroine (b.p. 60–70°). The yield of crude acid was 5.5 g; after the recrystallisations there remained 3.6 g. The acid forms microscopic needles with m.p. 84—86°.

0.1589 g acid: 15.84 ml 0.1065–*N* NaOH.
Equiv.wt. calc. 94.1 found 94.2
0.4773 g acid dissolved in absolute *ethanol* to 10.00 ml:
$2\,\alpha_D^{25} = +2.55°$. $[\alpha]_D^{25} = +26.7°$; $[M]_D^{25} = +50.3°$.

(—)-n-Pentylsuccinic acid. The brucine salt (41.3 g) was decomposed with dilute sulphuric acid and the organic acid was extracted with ether. It was recrystallised twice from ligroine and twice from water. The yield of crude acid was 6.7 g; after the recrystallisations there remained 5.2 g. M.p. 83–85°.

0.1509 g acid: 14.99 ml 0.1069–*N* NaOH.
Equiv.wt. calc. 94.1 found 94.2
0.4768 g acid dissolved in absolute *ethanol* to 10.00 ml:
$2\,\alpha_D^{25} = -2.505°$. $[\alpha]_D^{25} = -26.3°$; $[M]_D^{25} = -49.4°$.

0.5273 g acid dissolved in *water* to 10.00 ml:
$2\,\alpha_{D}^{25} = -2.16^\circ$. $[\alpha]_{D}^{25} = -20.5^\circ$; $[M]_{D}^{25} = -38.6^\circ$.

0.2579 g acid dissolved in *water* to 10.00 ml:
$2\,\alpha_{D}^{25} = -1.12^\circ$. $[\alpha]_{D}^{25} = -21.7^\circ$; $[M]_{D}^{25} = -40.9^\circ$.

As seen from the data for aqueous solution, the influence of the concentration on the rotatory power is rather pronounced.

Preparation of n-pentylsuccinic acid from 2-thenylsuccinic acid. 2.2 g. (0.01 mole) racemic 2-thenylsuccinic acid (3) and 3.0 g potassium carbonate were dissolved in a mixture of 350 ml water and 25 ml ethanol. Raney nickel (40 g) was added and the mixture refluxed for 45 minutes. The nickel was filtered off, and the filtrate evaporated to a small volume, acidified with sulphuric acid and extracted with ether. The yield of crude acid from the ether solution was practically quantitative. After one recrystallisation from water and two from hydrochloric acid, there remained 1.32 g, melting at 81–85°. A mixed sample with synthetic *n*-pentylsuccinic acid showed m.p. 81–86°.

0,1594 g acid: 15.81 ml 0.1069–*N* NaOH.
Equiv. wt. calc. 94.1 found 94.3

1.1 g (0.025 mole) (—)-2-thenylsuccinic acid and 1.5 g potassium carbonate were dissolved in 200 ml water +20 ml ethanol and refluxed with 20 g Raney nicke for 45 minutes. The acid was isolated as described above. The yield of crude product was 0.95 g; after recrystallisations from hydrochloric acid, ligroine and once more from hydrochloric acid there remained 0.62 g.

0.1714 g acid: 17.02 ml 0.1069–*N* NaOH.
Equiv. vt. calc. 94.1 found 94.2
0.3078 g acid dissolved in *water* to 10.00 ml:
$2\,\alpha_{D}^{25} = -1.34^\circ$. $[\alpha]_{D}^{25} = -21.8^\circ$; $[M]_{D}^{25} = -41.0^\circ$.

This value is in good agreement with the rotatory power found for the synthetic acid under corresponding conditions.

The author is indebted to Mr. K. PETTERSSON for assistance with part of the experimental work.

SUMMARY

By treatment with Raney nickel, optically active 2-thenylsuccinic acid can be converted into *n*-pentylsuccinic acid without racemisation. As the thenyl compound has previously been connected to the active benzylsuccinic acid and the configuration of the lower alkylsuccinic acids is known, we get the steric series illustrated by the formulas p. 278.

It is pointed out that this method can be of general use for connecting optically active aromatic compounds to the system of the glyceraldehyde.

The *n*-pentylsuccinic acid and its resolution into the optical antipodes are described

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala April 1953.

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Tryckt den 9 september 1953

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ARKIV FÖR KEMI Band 6 nr 25

Communicated 22 April 1953 by ARNE FREDGA

3-Thenylsuccinic acid

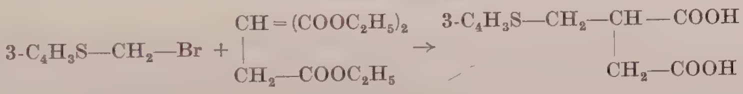
By SALO GRONOWITZ

With 1 figure in the text

The sidechain bromination of 3-methylthiophene with N-bromosuccinimide and benzoylperoxide as a catalyst by CAMPAIGNE and LESUER (1) opens a new route to 3-substituted derivatives of thiophene.

Therefore in connection with work in progress on optically active thiophene derivatives (2), the study of 3-substituted thiophenes has also been taken up and 3-thenylsuccinic acid has been prepared. The end in view is to resolve the acid into the optical antipodes and to compare them with the antipodes of benzylsuccinic acid and 2-thenylsuccinic acid.

3-Thenylsuccinic acid (I) has been prepared through a modified malonic ester synthesis from ethyl ethane-1.1.2-tricarboxylate (II) and 3-thenylbromide (III), followed by hydrolysis of the triester and decarboxylation.



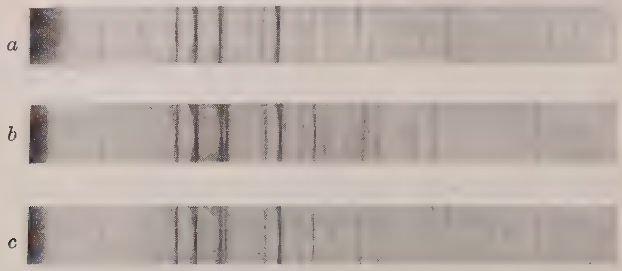
$$3\text{-}C_4H_3S{-}CH_2{-}Br + \begin{array}{l} CH{=}(COOC_2H_5)_2 \\ | \\ CH_2{-}COOC_2H_5 \end{array} \rightarrow \begin{array}{l} 3\text{-}C_4H_3S{-}CH_2{-}CH{-}COOH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2{-}COOH \end{array}$$

(III) (II) (I)

3-Thenylbromide has been prepared according to a modification of the original method of CAMPAIGNE and LESUER. As several authors have pointed out, it is difficult to get reproducible yields (3). Sometimes only nuclear bromination has been obtained by them. This indicates that the mechanism of the bromination with N-bromosuccinimide probably does involve radical intermediates, and the difficulty of obtaining reproducible results indicates that the radicaloid sidechain bromination is affected by small amounts of inhibitors. Recently CHAPMAN and WILLIAMS (4) have shown that the critical factor in the bromination of 2-methylnaphtalene was the purity of the N-bromosuccinimide. Using N-bromosuccinimide purified according to them, a yield of about 60% of 3-thenylbromide has been obtained. The experimental conditions of this reaction are under further investigation.

It is of some interest to study the phase relationships in the thiophene series. STEINKOPF (5) once claimed that isomorphism, which results in the tendency to form solid solutions is more frequent in the thiophene series than in other series of compounds. But recently COONRADT, HARTOUGH and JOHNSON (6) have shown that much of his evidence is incorrect.

The X-ray powder photographs of benzylsuccinic acid, 2-thenylsuccinic acid and 3-thenylsuccinic acid have been studied. The line distribution of the diffraction



a. Benzylsuccinic acid. *b.* 2-Thenylsuccinic acid. *c.* 3-Thenylsuccinic acid.

patterns is the same in the photographs, but the positions of the powder reflexions of benzylsuccinic acid (a) have smaller Bragg angels than those of the thenylsuccinic acids (b) and (c). This indicates that the acids are isomorphous and that the unit cell dimensions of benzylsuccinic acid are somewhat greater than those of the thenylsuccinic acids.

Preliminary thermal analysis of the systems benzylsuccinic acid–3-thenylsuccinic acid and 2-thenylsuccinic acid–3-thenylsuccinic acid have been undertaken according to the microscopical methods of Kofler (7). The contact preparations (8) confirm the formation of solid solutions. The phase diagrams will be published later. The system 2-thenylsuccinic acid–benzylsuccinic acid has been earlier studied by Fredga and Palm (2).

Experimental

3-Thenylbromide. In a three-necked flask, fitted with an efficient stirrer and a reflux condenser are placed a solution of 27.5 g (0.28 moles) of 3-methylthiophene in 75 ml of carbon tetrachloride. 44.3 g (0.25 moles) of N-bromosuccinimide, purified according to Chapman and Williams, 0.3 g of benzoylperoxide and four drops of water were added. The mixture was stirred and heated until a vigorous exothermic reaction began. After the initial reaction subsided, the mixture was refluxed for one hour. After cooling to 0° the lachrymatory reaction mixture was filtered. The carbon tetrachloride was removed *in vacuo*, and the residue rapidly distilled at 2 mm Hg. The fraction boiling between 50°–80° was used directly for the next step. It contains small amounts of 2-bromo-3-methylthiophene. Yield 36 g.

Ethyl 1.(3-thenyl) ethane-1.1.2-tricarboxylate. 3.9 g of sodium was dissolved in 150 ml of absolute alcohol and 40.0 g of ethyl ethane-1.1.2-tricarboxylate (9) was added. After cooling, 36 g of the bromination products were added dropwise with stirring, and the mixture was refluxed for six hours. The greater part of the alcohol was distilled off and the reaction product was washed with water. The ester was extracted with ether, dried and fractionated *in vacuo*. The fraction boiling between 160°–175° (2.5 mm Hg), was taken as ethyl 1.(3-thenyl) ethane-1.1.2-tricarboxylate. The pure ester boiled at 152° (1.5 mm Hg). The yield was 25.4 g.

3-Thenylsuccinic acid. The ester was saponified with 19.2 g of potassium hydroxide dissolved in the necessary amount of alcohol. After acidification with hydrochloric acid in excess the tricarboxylic acid was extracted with ether. After removing the ether, the acid was decarboxylated by refluxing with 75 ml of water for ten hours. On cooling the 3-thenylsuccinic acid crystallised as small white plates. The acid could be recrystallised from water. Yield 15.2 g (25% calculated on 3-methylthiophene). Using the microscopical method of Kofler (7), the melting point was 136°.

Anal. 0.02135 g: 4.45 ml 0.04475-*N* NaOH; — 0.01621 g: 0.02994 g CO_2 and 0.00642 g H_2O; — 0.03144 g: 0.01440 g H_2SO_4.

$C_9H_{10}O_4S$ (214.2)	calc.	Equiv. wt. 107.1	C 50.46	H 4.71	S 14.97
	found	Equiv. wt. 107.3	C 50.40	H 4.43	S 14.94

X-ray photographs

Powder photographs were taken in a focusing camera of the Guinier (10) type with CuK radiation (45 kV, 25 mA). Time of exposure six hours. The acids to be compared were obtained from evaporation of the same solvent (acetone).

Professor Arne Fredga had the kindness of putting benzylsuccinic acid and 2-thenylsuccinic acid at my disposal. I take this opportunity of expressing my sincere gratitude for this and for his valuable advice and help. I also want to express my gratitude to Fil. mag. Nils Karlsson for his expert advice in connection with the X-ray photographs.

Summary

3-Thenylsuccinic acid has been synthesised. X-ray powder photographs indicate that benzylsuccinic acid, 2-thenylsuccinic acid and 3-thenylsuccinic acid are isomorphous.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, April 1953.

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Struktur und Stabilität Schiff'scher Basen und ihrer Vorstufen

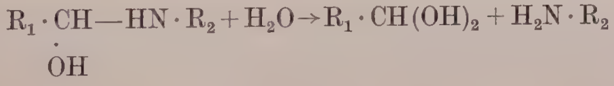
Von Hans von Euler, Hans Hasselquist und Olof Ceder

Mit 1 Figur im Text

Der Begriff „Schiff'sche Base" hat im Lauf der Jahre Änderungen erfahren, und seine verschiedene Umgrenzung hat noch in neuerer Zeit Anlass zu Diskussionen gegeben. Während man ursprünglich als Schiff'sche Basen nur die Kondensationsprodukte aromatischer Aldehyde mit primären Aminen bezeichnete, wurde dieser Begriff später immer mehr ausgedehnt, und zwar auf Produkte aus aliphatischen Aminen und aliphatischen Aldehyden und Ketonen.

Ganz abgesehen von einzelnen Produkten besonderer Zusammensetzung (wie z. B. Anhydro-Formaldehyd-Anilin) sowie von Polymerisaten aliphatischer Aldehyde mit aromatischen Aminen, hat man Stoffe angereiht, für welche die Struktur $R_1 \cdot CH = N \cdot R_2$ angenommen wurde, die sich aber bezgl. ihres Bildungsmechanismus und bezgl. ihrer Stabilität wesentlich von einander unterscheiden, und zwar sowohl durch die Lage als durch die Entwicklungsgeschwindigkeit des Gleichgewichtes mit ihren Komponenten.

Zieht man — wie in einer vorläufigen Mitteilung — die bei der Spaltung einer Schiff'schen Base, z. B. des Benzal-Anilins, eintretende Zwischenreaktion.



$$R_1 \cdot \underset{\displaystyle OH}{CH} — HN \cdot R_2 + H_2O \rightarrow R_1 \cdot CH(OH)_2 + H_2N \cdot R_2$$

in Betracht, so erkennt man, dass dieser Vorgang einer Hydrolyse analog ist. Das Zwischenprodukt $R_1 \cdot \underset{\displaystyle OH}{CH} — HN \cdot R_2$ kann demnach als Analogon eines Salzes einer sehr schwachen Säure und einer sehr schwachen Base betrachtet werden.[1]

Als Beispiele leicht spaltbarer „Schiff'scher Basen" mögen zunächst die bei der Formoltitration auftretenden Verbindungen zwischen Formaldehyd und Aminosäuren (H. Schiff, 1901; S. P. L. Sörensen, 1907) erwähnt werden. Die Gleichgewichte, die sich dabei in wenigen Sekunden einstellen, sind von der Azidität der Lösung abhängig.

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[1] Zur Kritik dieser Produkte siehe besonders die bemerkenswerte Arbeit von C. Prevost und A. Kirrmann, Bull. Soc. Chim. France (4) 49, 194, und 1309; 1931.

Auch die schon von Schiff[1] beschriebene Verbindung zwischen Glucose und Anilin wird schon in ganz schwach saurer Lösung (pH 5,60) schnell, bei pH 12,8 wesentlich langsamer hydrolysiert, wie uns Extinktionsmessung im Gebiet 210–300 mμ gezeigt haben.

Über den Einfluss von pH auf die Bildung und Spaltung von Schiffs Basen haben Haley und Maitland[2], hauptsächlich von biochemischen Problemen ausgehend, zahlreiche Versuche angestellt und sind u. a. zu folgendem Resultat gekommen: „In the reaction between an aldehyde and an amine in general the hydroxy-amine or Schiff base first formed may subsequently condense with one or other of the reactants, but, when both the aldehyde and the amine are aromatic, the highly crystalline stable Schiff bases are readily formed in high yield, and show no tendency towards the further reaction (Sprung, Chem. Rev. 1940, 26, 297).

In neuester Zeit zeigten Witkop u. Mitarb.[3] mit Hilfe des Infrarot-Spektroskops in einer bemerkenswerten Mitteilung, dass offene Schiff'sche Basen in wässriger Lösung schon von Spuren von Säuren in ihre Komponenten gespalten werden (wogegen sie nach Dimroth[4] bei Überschuss von HCl beständig sind) und sie wenden sich gegen Dimroths und Hantzschs[5] Auffassung, dass die sauren Salze Schiff'scher Basen sich von den hydratisierten Formen der Basen ableiten, dass sie also Ammoniumbasen der entsprechenden Carbinole seien.

Auch in rein aromatischen (eigentlichen) Schiff'schen Basen kann sich das Gleichgewicht verhältnismässig schnell einstellen. Dieser Vorgang wird nach Willi und Robertson[6] durch Säuren katalysiert. Versuche die Spaltung der aus Benzaldehyd und Anilin dargestellten Substanz (umkrystallisiert aus Alkohol, Schmpt. 51–52°) durch Papier-Chromatographie zu verfolgen, lieferten wegen ihrer hohen Löslichkeit in allen üblichen Lösungs-Systemen keine befriedigenden Resultate.

Spaltung der Schiff'schen Base aus Salicylaldehyd und Anilin[7]

Zur Darstellung des Salicyliden-Anilines wurden aequimolekulare Mengen der Komponenten auf dem Wasserbad kurz erwärmt. Aus der zunächst entstehenden gelben Flockung wurden durch Reiben Krystalle erhalten, die beim Umkristallisieren aus Petroleumäther den Schmpt. 51,5–52,5° zeigten. Dimroth und Zoeppritz (Ber. 35, 990; 1902), geben 50,5° an.

Die spektrophotometrischen Messungen beim Extinktionsmaximum 350 mμ zeigten dass nach Zusatz von Alkali (ca 0,01 n. NaOH) innerhalb 1 Stunde Spaltung in die Komponenten eingetreten war.

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[2] C. A. C. Haley und P. Maitland, J. Chem. Soc. 1951, 3155.

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[5] A. Hantzsch, Ber. 48, 1340; 1915. Die Angaben von Hantzsch (siehe auch Ber. 34, 822; 1901) über Benzaldehyd-p-chloranilin sind mehrfach als nicht zuverlässig kritisiert worden. Immerhin bestätigt sich, dass Benzyliden-p-Bromanilin in schwach saurer Lösung leicht gespalten wird.

[6] A. V. Willi und R. E. Robertson, 121st Meeting Amer. Chem. Soc. Abstracts, Milwaukee, April 1952.

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Papier-Chromatographie:

Wanderungs-flüssigkeit	pH	Anzahl Flecken und Farbe	Entwicklungs-flüssigkeit	R_f
80 Tle. Butanol 20 ,, Wasser 20 ,, Eisessig	3–4	1. Grün an der Grenze d. Wand. Flüss.	K-Bichromat + Schwefelsäure	—
Wasser gesättigt mit Butanol	6–7	1. Grün	,,	0,007
15 Tle. Butanol 130 ,, Wasser 5 ,, Ammoniak 25 %ig	10–11	Kein Flecken	,,	—

Auch der chromatographische Versuch zeigt also, dass Salicyliden-Anil in alkalischer Lösung gespalten wird: kein Flecken. (Das abgespaltene Anilin verflüchtigt sich im Verlauf der Chromatographierung.) In saurer Lösung ist hingegen das Salicyliden-Anil *stabil.* (Die Eisenchlorid-Reaktion beweist dass die phenolische OH-Gruppe im Anil wirklich intakt ist.) Durch diese Stabilität in saurer Lösung unterscheidet sich das Salicyliden-Anil wesentlich vom entsprechenden Anil der Zimtaldehydes wie die folgenden Messungen zeigen.

Quantitative Messungen über die Spaltung einer rein aromatischen Schiff'schen Base konnten am System *Zimtaldehyd-p-Bromanilin* ausgeführt werden und zwar durch Bestimmung der Extinktion der Lösungen im UV, da diese Schiff'sche Base eine kräftige Absorption bei 340 mμ zeigt, während die Absorption ihrer Komponenten im gleich Gebiet =0 oder äusserst gering ist.[1]

Das Zimtaldehyd-p-Bromanilin wurde aus den Komponenten in 96 %igem Alkohol in der Wärme dargestellt und durch Umkrystallisieren aus Äthanol in Form nadelförmiger Krystalle vom Schmpt. 121–122° erstmalig erhalten.

Spaltungsverlauf der Schiff'schen Base Zimtaldehyd-p-Bromanilin

Der zeitliche Verlauf der Extinktionsabnahme wurde bei verschiedenem pH gemessen und für jedes pH die Konstante der Spaltungsgeschwindigkeit ermittelt. Als Beispiele geben wir die Extinktionswerte E an, die für die bei pH 6,4, ferner 7,35 und 8,00 ausgeführten Versuche erhalten wurden (Temp. 22,5°, Konzentration der Schiff'schen Base: 244 mg/l).

Die für 8 pH-Werte gemessenen Reaktionskonstanten haben wir in Fig. 1 zusammengestellt. Dieselbe zeigt das sehr scharfe Maximum bei pH 8.

Der wesentliche Unterschied in der Stabilität der verschiedenen aromatischen Schiff'schen Basen, z. B. zwischen denen des Salicylaldehydes und des Zimtaldehydes beruht vielleicht darauf, dass Zimtaldehyd in wässriger Lösung viel rascher und

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[1] Versetzt man die alkoholische Lösung dieser Schiff'schen Base mit einer verdünnten Lösung einer Säure, so färbt sich die ursprünglich farblose Lösung gelb. Die gelbe Farbe verschwindet aber schnell wieder, und zwar um so schneller, je saurer die Lösung ist.

Vers. 1. pH 6,4		*Vers. 2.* pH 7,35		*Vers. 3.* pH 8,00	
Minuten	E	Minuten	E	Sekunden	E
0	1,54	0	1,50	0	0,81
1	1,40	1	1,21	10	0,72
2	1,29	2	1,00	20	0,64
3	1,19	3	0,87	30	0,58
4	1,10	4	0,73	40	0,53
5	1,00	5	0,65	70	0,39
7	0,86	6	0,58	90	0,33
10	0,67	10	0,45	120	0,25
15	0,48	13	0,20	180	0,17
20	0,36	18	0,38	240	0,13
Reakt.Konst. 0,036 Min^{-1}		Reakt.Konst. 0,076 Min^{-1}		Reakt.Konst. 0,280 Min^{-1}	

Zimtaldehyd-p-Bromanilin

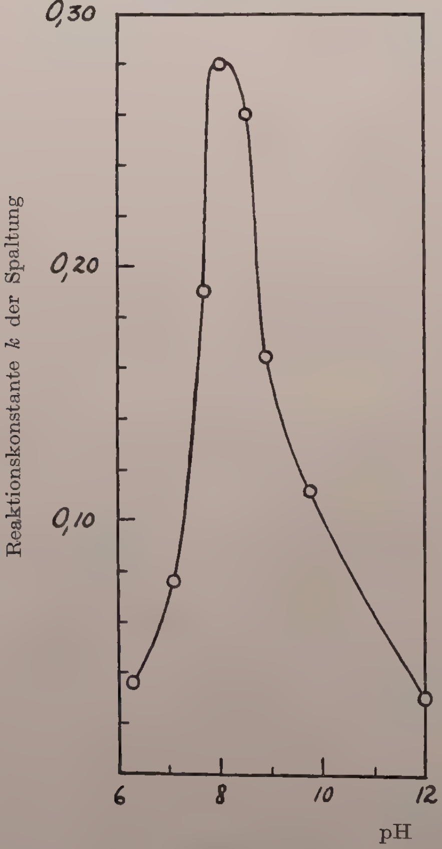


Fig. 1.

vollständiger in die Vorstufe $R_1\cdot\underset{\displaystyle OH}{CH}-HN\cdot R_2$ übergeht, welche dem in der Figur dargestellten Versuch zugrunde liegt.

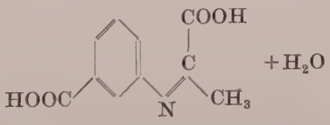
Wir gehen — bezugnehmend auf das S. 287 und in der vorläufigen Mitteilung[1] (l. c.) Gesagte — davon aus, dass die Vorstufe der Schiff'schen Base sich im Prädissoziationszustand befindet, und zwar an der Stelle $-\underset{\displaystyle OH}{CH}-HN-$, an welcher die Oscillation zwischen der Bindung durch ein Elektronenpaar und dem ionisierten Zustand statthat. Unsere Problemstellung berürhrt den Zusammenhang zwischen dem Atomabstand C–N bezw. der Oscillationsgrösse dieses Atompaares in der besprochenen Vorstufe und dem Übergang dieser Vorstufe in die beiden Ionen.

Wir suchen noch weitere experimentelle Unterlagen für die Annahme, dass bei der Spaltung der Vorstufen der Schiff'schen Basen Elektronenpaare oszillatorisch in Ionenladungen übergehen (eine Prädissoziation erfahren) bezw. dass in wässriger Lösung ein elektromeres Gleichgewicht besteht zwischen dem durch ein Elektronenpaar verbundenen Molekül $R\cdot\underset{\displaystyle OH}{CH}-HN\cdot R_1$ und dem nach Trennung der chemischen Bindung auftretenden Ionenpaar.

Wir haben im Vorhergehenden zwei Typen von aromatischen Schiff'schen Basen besprochen, von denen der eine sowohl in saurer als in alkalischer Lösung sehr leicht in die Komponenten zerlegt wird, während der andere in saurer Lösung stabil, in alkalischer Lösung labil ist.

Schiff'sche Basen vom zweiten Typus sind im Kreis unserer früheren Untersuchungen gelegen, und zwar

a) Produkte aus Brenztraubensäure und Aminobenzoesäuren. Von den früher von uns untersuchten Stoffen[2] wurde neuerdings der durch Formel (1) dargestellte auf seine Spaltbarkeit in saurem und alkalischem Medium untersucht.



(1)

Diese Substanz ist sowohl in saurer wie in alkalischer Lösung *stabil.*

b) Redukton-Derivate, nämlich Produkte aus Triose-Redukton und aromatischen Basen (Anilin u. a.). Diese Stoffe sind sowohl hier[3] als im Arbeitskreis von Cocker und O'Meara[4] untersucht worden. Wir geben hier die Ergebnisse eines papierchromatographischen Versuches an, den wir an Redukton-Anil (Schmpt. 119°) angestellt haben.

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[1] Bald nach Erscheinen dieser Mitteilung haben G. Carpéni u. J. Chouteau (C. r. Acad. Sci. (Paris) 236, 1354; 30. März 1953) eine ganz ähnliche Problemstellung behandelt, auf die wir bald zurückkommen.

[2] Euler, H. Hasselquist u. E. Eriksson, Dieses Arkiv 5, Nr 23; 1953.

[3] Euler, H. Hasselquist u. U. Lööv, Arkiv för Kemi etc. 26 A Nr 17; 1948.

[4] W. Cocker, R. A. O'Meara, J. C. P. Schwarz u. E. R. Stuart, J. Chem. Soc. 1950, 2052. — B. Eistert u. H. Hasselquist, Dieses Arkiv 4, Nr. 12; 1952.

Wanderungsflüssigkeit	pH	Anzahl Flecken Farbe	Entwicklungslösung	R_f
80 Tle. Butanol 20 „ Wasser 20 „ Eisessig	3-4	a) 1, schwarz b) 1, grün	Silbernitrat+ NH_3 K-bichromat + H_2SO_4	0,94
15 Tle. Butanol 130 „ Wasser 3 „ Ammoniak 25 %ig	10–11	a) 1, graugrün bei 30 min. Chromatogr.-dauer b) keine Flecken bei Chromatogr.-dauer 30 Min.	„ oder $AgNO_3$	0,79

Das Redukton-Anil ist also in saurem Medium stabil, in alkalischem Medium wird es bei längerem Verweilen im papierchromatographischen Apparat gespalten, vielleicht indem eine Oxydation zu Mesoxaldialdehyd-mono-anil eintritt, das leicht spaltbar sein kann.

Abgesehen von den verschiedenen oben erwähnten Typen der Schiff'schen Basen erstreckt sich unsere Problemstellung auf andere grosse Klassen von Substanzen, unter denen die Verbindungen der Aldehyde mit *Hydrazinen* und mit *Säureamiden* hervorgehoben seien.

Die Einwirkung von Aldehyden auf Säureamide führt zu verschiedenen Produkten[1], unter denen uns im vorliegenden Zusammenhang besonders die mit substituierten Aldehyden entstehenden Verbindungen vom Typus $R_1 \cdot \underset{\displaystyle OH}{\underset{\cdot}{CH}}—HN \cdot CO \cdot R_2$ interessieren, die der Vorstufe der Schiff'schen Basen entsprechen.

Stockholm, Universität, Institut f. organ. chemische Forschung.

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[1] Siehe hierzu A. REICH, Monatshefte f. Chemie, 25, 933; 1904. — Ferner B. M. GUPTA, J. Chem. Soc. (London) 119; 298, 1921.

Tryckt den 9 september 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 27

Mitgeteilt am 3. Juni 1953 durch A. WESTGREN und J. ARVID HEDVALL

Untersuchung des Einflusses der Luftfeuchtigkeit auf Mahl- und Separierungsvorgänge bei festen Stoffen

Von ÅKE ALBINSSON

Mit 8 Figuren im Texte

Das Ziel der vorliegenden Untersuchung war die Aufklärung der Bedeutung der relativen Luftfeuchtigkeit auf den Verlauf der Mahl- und Separierungsvorgänge von festen Stoffen. Eine Behandlung dieser Fragen ist von grösster praktischer Bedeutung für die Zerkleinerung und Separierung fester Stoffe in der Industrie wie im Laboratorium. Beispiele dafür sind u. a. der Einfluss der Luftfeuchtigkeit auf die Materialseparierung nach der Siebanalyse, das Zusammenbacken von Pulvern beim Lagern in feuchten Wetter sowie alle Fragen der Entstaubung von Lokalen.

Die Theorie der Zerkleinerung grösserer Aggregate spröder Stoffe wurde in zusammenfassender Weise von SERKAL[1] behandelt; ausgedehnte Untersuchungen über die Zermahlungsvorgänge wurden von HÜTTIG angestellt.[2]

Der Einfluss der umgebenden Gasatmosphäre auf die Eigenschaften fester Stoffe wurde im letzter Zeit grössere Aufmerksamkeit geschenkt seit HEDVALL nicht nur die Einwirkung chemisch wirksamer Gase wie SO_2, CO_2 usw. sondern auch im gewöhnlichen Sinne inaktiver Gase auf das Verhalten fester Stoffe aufzeigen konnte.[3] Bezüglich des hier speziell interessierenden Einflusses von Wasserdampf liegen Untersuchungen über die Einwirkung desselben auf keramische Stoffe (SANDFORD[4]) und das Rohmaterial der Glasfabrikation vor (FRÖLICH[5]).

Der Arbeitsplan der vorliegenden Untersuchung umfasste eine Bestimmung der Separierungsgeschwindigkeit nach Korngrössen der Stoffe Quarz, Feldspat und Kaolin nach der Methode der Siebananalyse, d. h. in Laboratoriumskala und eine Bestimmung der Mahl- und Separierungsgeschwindigkeit von Feldspat in industrieller Skala. Anschliessend wurde der Einfluss der Korngrössenverteilung derart hergestellter Feldspatpulver auf die Einschmelzung von Feldspat im Glasfluss behandelt.

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[1] A. SMEKAL und W. KLEMM, Monatsh. Chemie *82* (1951), 411 und dort angegebene Literatur.

[2] G. F. HÜTTIG, Dechema-Monographien 21 (1952), 96.

[3] J. A. HEDVALL u. T. GÜNTHER, Z. anorg. allg. Chem. 251 (1943) 3. 0. 5; J. A. HEDVALL u. A. LUNDBERG, Kolloid-Zeitschr. 104 (1943), 198; J. A. HEDVALL u. M. BLOMQVIST, Arkiv kemi, min. geol. (A) *19* (1944) Nr 22.

[4] J. F. SANDFORD, Chalmers Tekn. Högskolas Handl. Nr 36 (1944).

[5] Å. FRÖLICH u. J. A. HEDVALL. Chalmers Tekn. Högskolas Handl. Nr 31 (1944).

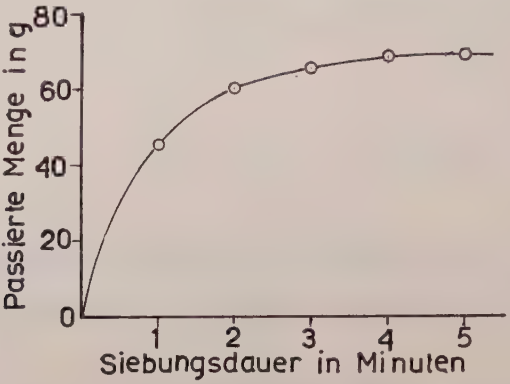

Fig. 1. Zusammenhang zwischen Siebungsdauer und passierter Menge Feldspat von Forshammar (Korngrösse Din 80 bis 100).

A. Separierungsgeschwindigkeit beim Sieben von Pulvern in Abhängigkeit von der Luftfeuchtigkeit

1. Versuchsanordnung

Das Versuchsmaterial wurde in einer Kugelmühle gemahlen und geeignete Fraktionen ausgesiebt. Folgende Korngrössenfraktionen wurden gewählt:

I. Kleiner als Din 100 (d. h. kleiner als 0,06 mm).
II. Zwischen Din 80 und Din 100 (Korndurchmesser 0,06 bis 0,075 mm).
III. Zwischen Din 60 und 80 (Korndurchmesser 0,075 bis 0,102 mm).

75 g des gemahlenen Materials wurden in einem Exsickator über entsprechenden Schwefelsäurelösungen einer Atmosphäre mit gewünschter Luftfeuchtigkeit ausgesetzt. Der Exsickator befand sich dabei in einem Luftthermostat in welchem die Temperatur mittels Wärmeelement und Temperaturregulator auf 25° gehalten werden konnte. Die Proben befanden sich in Wägegläsern und wurden von Zeit zu Zeit gewogen bis konstantes Gewicht festgestellt werden konnte. Sobald die Proben mit der Luftfeuchtigkeit im Gleichgewicht waren, wurde das Material in einer Schüttelanordnung unter 5 Minuten gesiebt und die passierte Menge gewogen. Die Siebdauer wurde so kurz gewählt um die Verstopfung des Siebes so weit als möglich auszuschalten. Der Zusammenhang zwischen Siebdauer und passierter Menge wurde bestimmt und in Fig. 1 graphisch wiedergegeben. Aus dieser Kurve geht hervor, dass die Separierungsgeschwindigkeit unmittelbar nach Beginn rasch abnimmt um nach etwa 5 Minuten einen ziemlich konstanten Wert anzunehmen. Das zu diesem Vorversuch verwendete Material war Feldspat mit Korngrössen zwischen 0,06 und 0,075 mm.

2. Versuchsergebnisse

In den folgenden Tabellen wird das Ergebnis der Bestimmung der in 5 Minuten passierten Mengen in Abhängigkeit von der Luftfeuchtigkeit wiedergegeben. Die zur Separierung gebrachten Mengen waren durchgehend, wie oben erwähnt, 75 g.

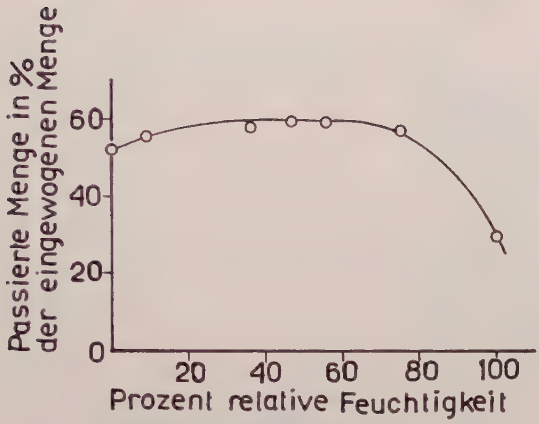

Fig. 2. Zusammenhang zwischen Separierungsgeschwindigkeit und relativer Luftfeuchtigkeit für Quarzsand (Korngrösse Din 80 bis 100).

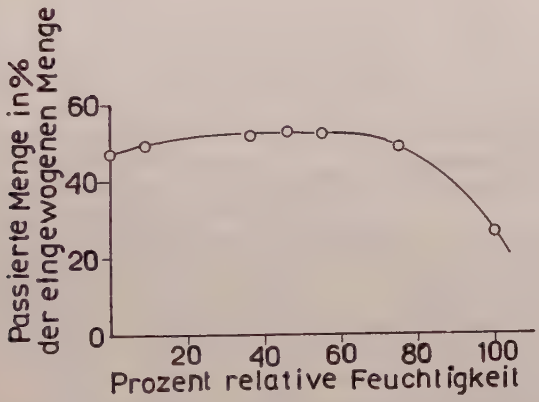

Fig. 3. Zusammenhang zwischen Separierungsgeschwindigkeit und relativer Luftfeuchtigkeit für Quarzsand (Korngrösse Din 60 bis 80).

Tabelle 1. Quarz

Relative Luftfeuchtigkeit in Prozent	Passierte Menge des Materials in Prozent der eingewogenen Menge bei Korngrössen von	
	Din 80–100	Din 60–80
0	52,7	47,0
35	58,9	52,0
46	60,0	53,4
75	58,7	49,7
100	30,0	27,0

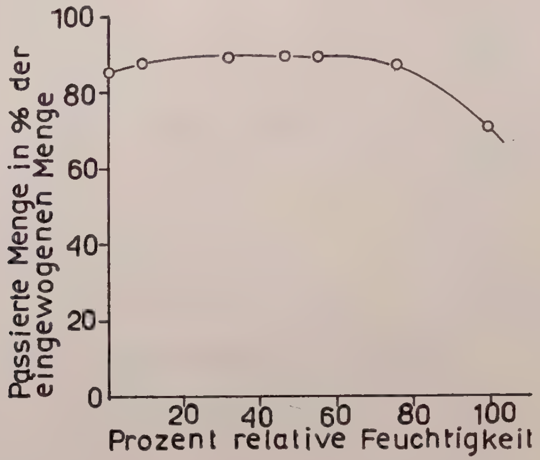

Fig. 4. Zusammenhang zwischen Separierungsgeschwindigkeit und relativer Luftfeuchtigkeit für Feldspat (Korngrösse Din 80 bis 100).

Tabelle 2. Feldspat

Relative Luftfeuchtigkeit in Prozent	Passierte Menge des Materials in Prozent der eingewogenen Menge bei Korngrössen von	
	Din 80–100	Din 60–80
0	85,0	82,0
9	88,7	85,5
36	89,4	87,0
46	89,4	87,0
75	87,0	83,0
100	70,0	53,5

Tabelle 3. Chamotte

Relative Luftfeuchtigkeit in Prozent	Passierte Menge des Materials in Prozent der eingewogenen Menge. Korngrösse Din 80–100
0	52,0
9	53,0
36	55,0
46	55,0
55	53,0
75	53,0
100	38,0

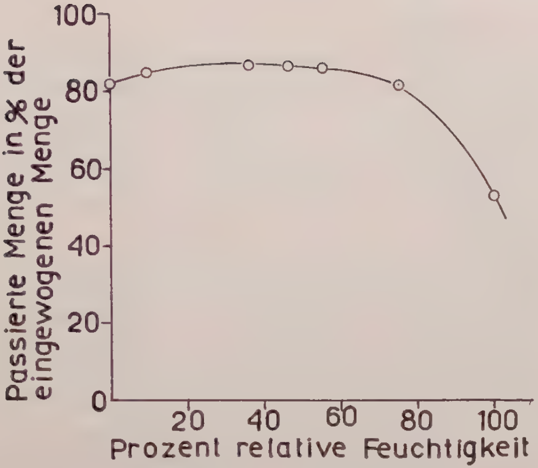

Fig. 5. Zusammenhang zwischen Separierungsgeschwindigkeit und relativer Luftfeuchtigkeit für Feldspat (Korngrösse Din 60 bis 80).

Der Verlauf wird für die Korngrössen Din 80–100 in Figur 2 und für die Korngrössen Din 60-80 in Fig. 3 graphisch wiedergegeben wobei der prozentuelle Durchgang auf der Ordinate und die relative Luftfeuchtigkeit auf der Abszisse aufgetragen wurde.

Die beiden Kurven zeigen ein Maximum zwischen 40 und 60 Prozent relativer Luftfeuchtigkeit und fallen bei hohen Feuchtigkeitsgraden stark ab. Die Verschlechterung der Siebungsausbeute bei geringerer Feuchtigkeit als der optimalen ist wesentlich weniger ausgeprägt.

Die Abhängigkeit der Separierungsgeschwindigkeit wurde in Fig. 4 (Korngrösse Din 80–100) und Fig. 5 (Korngrösse Din 60–80) graphisch wiedergegeben. Das Ergebnis der Separierungsbeeinflussung mit einem Maximum bei mittlerer Luftfeuchtigkeit ist in Übereinstimmung mit den an Quarz erhaltenen Resultaten.

3. Untersuchung der Ursache der geringeren Separierungsgeschwindigkeit bei trockenen Proben.[1]

Entsprechend der Annahme, dass die geringere Separierungsgeschwindigkeit bei trockenen Proben auf ein Ladungsphänomen zurückzuführen sei, wurden folgende Versuche angestellt:

Während der Separierung wurde oberhalb des Siebes eine Quarzlampe angebracht um durch deren UV-Licht die Luft zu ionisieren und eventuell durch Entladung der Körner den beobachteten „Trocken-Effekt“ zu beeinflussen. Eine Erhöhung der Separierungsgeschwindigkeit konnte dadurch jedoch nicht erzielt werden — vermutlich weil die Ionisierung durch UV-Strahlung zu gering ist. Darauf kann aus der Tatsache geschlossen werden, dass folgende Anordnung mit stärkerer Ionisierung die obige Annahme vollauf bestätigte: nach Versetzen von Feldspatpulver mit Korngrössen zwischen Din 80 und Din 100 mit einer Thorium-B-Lösung, Eindunstung des Präparats und nachfolgender Behandlung mit Luft verschiedener Feuchtigkeit

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[1] Å. Albinsson, Arkiv kemi, min. geol., (B) *26* (1948), Nr. 7.

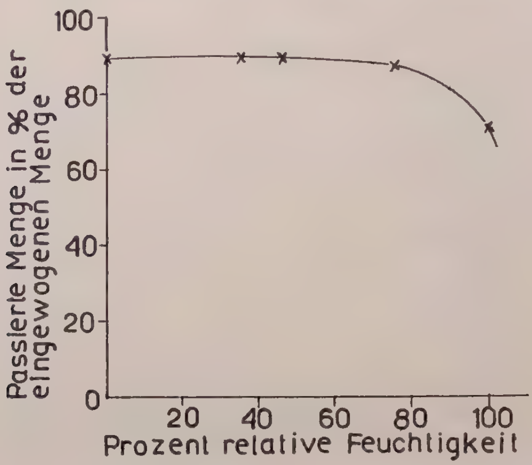

Fig. 6. Zusammenhang zwischen Separierungsgeschwindigkeit und relativer Luftfeuchtigkeit für Thorium B-behandeltem Feldspat (Korngrösse Din 80 bis 100).

wie oben beschrieben, verschwand der „Trocken-Effekt" d. h. die Siebungsgeschwindigkeit war, wie Fig. 6 zeigt, zwischen Null und 40 % Luftfeuchtigkeit gleich gross. Erst oberhalb etwa 75 % Luftfeuchtigkeit trat auch in dem mit Th B behandelten Präparat die bereits mehrmals beschriebene starke Verminderung der Separierungsgeschwindigkeit ein, welche offenbar auf eine Zusammenbackung des Pulvers zufolge der Kapillarkondensation des Wassers im Pulver zurückzuführen ist.

B. Untersuchung des Einflusses der Feuchtigkeit auf das Mahlen und Separieren von Feldspat in industrieller Skala

Das Ziel der in diesem Abschnitt beschriebenen Untersuchungen war die Aufklärung der Bedeutung der Feuchtigkeit von Mahlgut für die Geschwindigkeit der Zerkleinerung und Separierung desselben in technischer Skala. Ausserdem wurde auch der Einfluss der Feuchtigkeit auf die beim Zerkleinern erhaltene Korngrössenteilung studiert.

1. Versuchsanordnung

Diese Versuche in grosser Skala wurden mit einer Kugelmühle in einer grossen Fabrik durchgeführt. Durch die Mühle wird ein Luftstrom geleitet, dessen Geschwindigkeit gerade so gross ist, dass Partikeln unter einer gewissen Korngrösse mitgeführt werden. Der Luftstrom passiert darauf einen Zyklon in welchem das Pulver grösstenteils abgeschieden wird. Der Rest, d. h. die feinsten Feldspatkörner werden darauf in einer Staubkammer, in welcher die Luft gezwungen wird aufgehängte Säcke zu passieren, abgestezt.

Die Versuche wurden so durchgeführt, dass der grobzerkleinerte Feldspat vor dem Einsatz in die Mühle mit Wasser versetzt und die Feuchtigkeit des Malguts an Hand von der Mühle entnommenen Proben bestimmt wurde. Die im Zyklon erhaltenen Mengen gemahlenen Feldspats sowie die Korngrössenverteilung des Pulvers wurde daraufhin während der ganzen Operation kontrolliert. Das trockenste Material in dieser Versuchsserie wurde so erhalten, dass grobe Stücke Feldspat an der Sonne getrocknet, darauf grobzerkleinert und unmittelbar nachher in die Kugelmühle gebracht wurden. Die Feuchtigkeit dieser Probe wurde zu 0,05 % der eingebrachten Menge bestimmt und dürfte den praktischen Grenzfall darstellen. Grobzerkleinerter Feldspat, der unter längerer Zeit gelagert wurde und dabei ins Gleichgewicht mit der im Lokal herrschenden Luftfeuchtigkeit kam, hatte wesentlich höheren Feuchtigkeitsgehalt.

Durch die Friktion zwischen Material und Wänden der Kugelmühle, sowie durch die Schläge der Kugeln gegen die Wände tritt eine beachtliche Temperaturerhöhung in der Kugelmühle ein. Diese ist ausser dem Einfluss der Kornverteilung und Kornform abhängig vom Feuchtigkeitsgrad des Guts. Bei einer Feuchtigkeit des Materials von 0,05 % wurde in der Mühle eine Temperatur von 49° C gefunden, während diese bei einem Feuchtigkeitsgehalt von 0,23 % nur 32° C war. Die Temperatur in der Kugelmühle ist von grösster Bedeutung für die Kapazität der Anlage und bestimmt die Temperatur der zirkulierenden Temperatur und damit deren Vermögen Feuchtigkeit aufzunehmen und wegzuführen.

2. Versuchergebnisse

In der folgenden Tabelle 4 wird die Kapazität der verwendeten Zerkleinerungsanlage, ausgedrückt in kg feinzerkleinertern Feldspats per Stunde, in Abhängigkeit von der Feuchtigkeit des Malguts wiedergegeben. Bezüglich der Korngrössenverteilung der Pulver wird auf Tabelle 8 hingewiesen.

Tabelle 4

Feuchtigkeit des Materials in Prozent	Kapazität der Anlage in kg gemahlenen Feldspats im Zyklon per Stunde
0,05	550
0,09	425
0,16	340
0,23	300

Fig. 7 gibt diesen Zusammenhang zwischen Feuchtigkeit des Materials und Kapazität der Mühle graphisch wieder. Wie aus dieser und aus Taballe 4 hervorgeht, verursacht bereits eine geringe Erhöhung des Feuchtigkeitsgehaltes eine markante Erniedrigung der Kapazität der Kugelmühle, verursacht durch ein Zusammenbacken des feinkörnigen Materials, welches die noch groben Stücke umhüllt und dadurch den Stoss der Kugeln dämpft. Ausserdem vurursacht dieses Phänomen noch ein weiteres Übel, nämlich dass grössere Körner durch den Luftstrom aus der Mühle mitgerissen werden. Eine weitere Gefahr in dieser Hinsicht ist ferner dadurch gegeben, dass man versucht ist die Ausbeute durch eine erhöhte Luftzufuhr zu vergrössern und dadurch die Gefahr des Mitreissens gröberer Feldspatkörner noch weiter vergrössert.

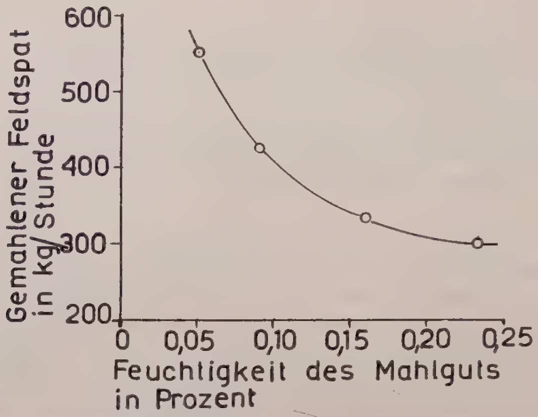

Fig. 7. Zusammenhang zwischen Mühlenkapazität und Feuchtigkeit des Mahlguts (Versuch ohne Sieb in der Kugelmühle).

Um ein Mitreissen grösserer Feldspatkörner zu verhindern, wurde vor den folgenden Versuchen in der Kugelmühle, etwa 3 dm vor dem Auslauf ein Siebtuch mit 3 mm Maschenweite angebracht. Das Sieb wurde durch ein vor demselben angebrachten Drahtnetz vor aufprallenden Kugeln oder grösseren Feldspatstücken geschützt. Nach Bestimmung des geeignetesten Füllungsgrades der Mühle konnte daraufhin die Geschwindigkeit des Luftstroms erhöht werden ohne ein Mitreissen grösserer Feldspatstücke befürchten zu brauchen. Nach dieser Veränderung der Zerkleinerungsanlag konnte eine Kapazitätserhöhung von 40 % festgestellt werden. Diese ist darauf zurückzuführen, dass die Korngrössenverteilung des erhaltenen Pulvers in der Richtung nach gröberen Fraktionen verschoben wurde, wobei allerdings infolge des Siebes die maximale Korngrösse theoretisch auf 3 mm beschränkt blieb. Eine Kontrolle der tatsächlichen maximalen Korngrenze ergab etwa 1,5 mm.

Eine Bestimmung der Kapazität der Kugelmühle in Abhängigkeit von der Feuchtigkeit des Mahlguts nach Anbringung des Siebes ergab die in Tabelle 5 wiedergegebenen Werte.

Tabelle 5

Feuchtigkeit des Materials in Prozent	Kapazität der Anlage in kg gemahlenen Feldspats im Zyklon per Stunde
0,1	700
0,2	600
0,5	550
1,0	450

Bei einem Vergleich zwischen den Ergebnissen der früheren Versuchsserie ohne Sieb und diesen mit Sieb (vgl. auch Fig. 8) zeigt es sich, dass der Feuchtigkeitsgehalt des Guts in dem letzteren Fall einen geringeren Einfluss auf die Kapazität der Mahlanlage ausübt. Dieses Ergebnis kann dadurch erklärt werden, dass bei der

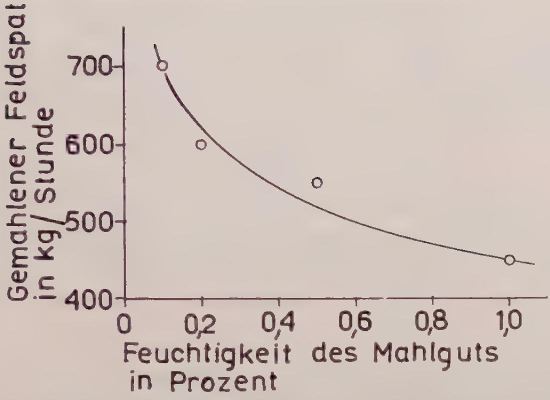

Fig. 8. Zusammenhang zwischen Mühlenkapazität und Feuchtigkeit des Mahlguts (Versuch mit Sieb in der Kugelmühle).

Anwendung der Siebanordnung in der Mühle, die Luftzirkulation grösser und die Mühle weniger gefüllt ist, wodurch die Korngrössenverteilung gegen gröbere Fraktionen verschoben wird. Es gibt also in diesem Fall eine geringere Menge feinsten Pulvers, welches wie oben erwähnt durch Zusammenbackung die Mahlausbeute erniedrigt.

3. Zusammenhang zwischen der Feuchtigkeit des Mahlguts und der Korngrössenverteilung des gemahlenen Feldspats

Wegen der Bedeutung der Korngrössenverteilung von Pulvern auf deren Mischbarkeit mit anderen, wie in dem hier interessierenden Feldspatpulver mit Kalk, Sand und Soda für die Glasherstellung, wurde auch der Einfluss der Feuchtigkeit auf die Korngrössenverteilung des in der Kugelmühle gemahlenen Feldspates untersucht.

Theoretisch gilt für die geeigneste Korngrössenverteilung für ein gegebenes Gemenge unter Annahme runder Körner

$$\frac{a_2}{a_1} = \sqrt[3]{\frac{p_2 \cdot d_1}{p_1 \cdot d_2}}.$$

Diese Formel wird hergeleitet unter der Annahme, dass es für jedes Korn mit der Korngrösse a_1 mm des Materials A_1 pro Menge p_1 kg ein Korn des Materials A_2 in der Menge p_2 kg von der Korngrösse a_2 mm geben muss um idealische Mischbarkeit zu erhalten. Wenn die Anzahl Körner in der Menge p_1 kg mit n_1 und in der Menge p_2 kg mit n_2 bezeichnet wird, dann erhält man:

$$n_1 = \frac{p_1}{\frac{1}{6}\, n \cdot a_1^3 \cdot d_1} - = n_2 = \frac{p_2}{\frac{1}{6}\, n \cdot a_2^3 \cdot d_2}$$

woraus folgt

$$\frac{a_2^3}{a_1^3} = \frac{p_2 \cdot d_1}{p_1 \cdot d_2}.$$

Bei der Berechnung der theoretisch besten Mischbarkeit eines Gemenges geht man von der gegebenen Korngrössenverteilung des Sandes aus und gruppiert die Korngrössen der übrigen Komponenten um diese. In dem hier untersuchten Fall handelte es sich um Skåne-Sand mit der in Tabelle 6 wiedergegebenen Korngrössenverteilung.

Tabelle 6

Maschenweite in mm	Nicht passierte Menge in Prozent der gesamten Menge
0,6–0,5	1,0
0,5–0,3	21,5
0,3–0,15	73,5
0,15	4,0

Die theoretisch geeignetste Korngrössenverteilung für den Feldspat kann danach mit Hilfe oben angegebener Formel berechnet werden. Diese wurde in Tabelle 7 zusammengestellt.

Tabelle 7

Maschenweite in mm	Nicht passierte Menge in Prozent der gesamten Menge
0,4 –0,33	1,0
0,33–0,2	21,5
0,2 –0,1	73,5
0,1	4,0

Bei der Zermahlung von Feldspat in der Kugelmühle unter Anwendung des oben beschriebenen Siebtuches mit einer Maschenweite von 3 mm wurden bei den Feuchtigkeitsgehalten des Mahlguts von 0,1, 0,2 und 1,0 % die in Tabelle 8 wiedergegebenen Kornverteilungen bestimmt.

Tabelle 8

Maschenweite in mm	Nicht passierte Menge in Prozent der gesamten Menge bie den Feuchtigkeitsgehalten in Prozent		
	0,1	0,2	1,0
0,6	4	6	10
0,6 –0,3	10	17	24
0,3 –0,15	23	31	32
0,15–0,1	13	13	9
0,1 –0,07	9	7	5
0,07	41	26	20

Der Vergleich zwischen der theoretisch berechneten und den bei den Versuchen erhaltenen Korngrössenverteilungen zeigt, dass die letzteren zuviel der Fraktionen unter 0,1 und über 0,3 mm enthalten. Um diesem Umstand abzuhelfen, wurden statt des früher verwendeten Siebtuchs mit 3 mm Maschenweite solche mit 2 und 1,5 mm Maschenweite in die Mühle eingesetzt. Es zeigte sich da aber, dass durch diese Massnahme allein die maximale Korngrenze der Körner herabgesetzt wurde, während die Siebanalysen im übrigen unberührt blieben.

Zur Bestimmung der geeignetsten Maschenweite des Siebes in der Mühle wurde daher rein empirisch untersucht, welche Gemische bei der Erschmelzung von Glas am raschesten geläutert werden. Über diese Versuche wird in dem folgenden, letzten Abschnitt berichtet.

C. Läuterung von Glasschmelzen aus Gemengen unter Beimischung von Feldspat verschiedener Korngrössenverteilung

1. Versuchsführung

Feldspat verschiedener Korngrössenverteilung wurde mit Soda, Sand und Kalk gemischt und das so erhaltene Gemenge in Quantiteten von 50 g in Chamotte-Tiegeln in einem ölgeheitzten Ofen während 3 Stunden erschmolzen. Die Zusammensetzung des Gemenges war dabei in allen Fällen

Sand	435	g
Soda	167	»
Kalk	125	»
Feldspat	56	»

Die erkalteteten Glasschmelzen wurden darauf unter dem Mikroskop an verschiedenen Stellen auf die Anzahl noch vorhandener Blasen untersucht und diese Zahl als Mass für den Läuterungsgrad und damit für die Eignung der entsprechenden Korngrössenverteilung des Feldspats angesehen.

Folgende Korngrössenverteilungen des Feldspatpulvers wurden in diese Weise untersucht:

I. Feldspatpulver mit einer Verteilung entsprechend der theoretisch berechneten für beste Mischbarkeit.
II. Fraktion zwischen 0,1 und 0,2 mm.
III. Mahlprodukt bei einer Maschenweite des Siebes in der Mühle von 1,5 mm.
IV. Mahlprodukt bei einer Maschenweite des Siebes in der Mühle von 2,0 mm.
V. Mahlprodukt bei einer Maschenweite des Siebes in der Mühle von 3,0 mm.
VI. Feldspatpulver aus der oben erwähnten Abscheidekammer hinter dem Zyklon.

2. Versuchsresultate

Die Untersuchung der Blasenanzahl in den verschiedenen Schmelzen ergab mit einer Abweichung von etwa 10 % das gleiche Resultat für die Schmelzen I, II, III, IV und VI, nähmlich ungefähr 265. Bei Schmelze V mit den grössten Feldspatkörnern war die Blasenanzahl grösser, nämlich 356, und lässt dieses Produkt daher als weniger geeignet erscheinen. Nachdem Feldspat mit Kornverteilungen entsprechend I und II praktisch nicht in Frage kommen und bei Siebtüchern mit einer Maschenweite von 1,5 mm die Kapazität der Mühle geringer ist als bei einer Maschenweite von 2,0 mm erscheint diese als die geeignetste.

D. Zusammenfassung der Versuchsergebnisse

Die Separierungsgeschwindigkeit von Quarz-, Feldspat- und Kaolinpulver ist abhängig von der Luftfeuchtigkeit und erreicht maximale Werte zwischen etwa 40 und 60 % relativer Luftfeuchtigkeit. Oberhalb 75 % Luftfeuchtigkeit fällt die Separierungsgeschwindigkeit stark ab zufolge Kapillarkondensation und damit verbundenem Zusammenbacken der Pulver.

Bei niedrigen Werten der Luftfeuchtigkeit ist die Separierungsgeschwindigkeit ebenfalls geringer. Als Ursache für diese Abweichung wurde eine elektrische Aufladung der Partikeln gefunden. Diese kann eliminiert werden durch starke Ionisation der Luft, z. B. mittels radioaktiver Strahlung.

Ein markanter Einfluss der Feuchtigkeit wurde auch auf Geschwindigkeit und Verlauf der Zermahlung von Feldspat festgestellt. Im Gegensetz zu den Separierungsvorgängen ist der Einfluss auf die Zermahlung einfacher und äussert sich in einer stetigen Abnahme der Mühlenkapazität mit steigender Feuchtigkeit des Mahlguts. Die Ursache dieser Erscheinung ist eine Einhüllung der gröberen Stücke durch feineres Pulver und die damit verbundene Dämpfung der Kugelstösse. Damit verbunden werden Variationen der Korngrössenverteilungen in Abhängigkeit von der Feuchtigkeit des Guts festgestellt.

Die für eine rasche Einschmelzung von feldspathaltigen Glassätzen geeignetste Korngrössenverteilung von Feldspat wurde bestimmt.

Für die Durchführung dieser Untersuchungen wurden dem Vorstand des Instituts für Silikatforschung Mitteln von Statens Tekniska Forskningsråd zur Verfügen gestellt.

Herrn Professor J. A. HEDVALL danke ich für sein stetes Interesse an der Ausführung dieser Arbeit.

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Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 28

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Use of ion exchangers for the analysis of reaction mixtures. The alkaline hydrolysis of cyanoacetamide

By SIGVARD WIDEQVIST

Recently the author (1) described a convenient method for following the alkaline hydrolysis of amides. Samples of the reaction mixture were allowed to flow through a cation exchanger containing Amberlite IR 120 and titrated with standardized sodium hydroxide. In this very simple way the amount of carboxylic acid, x, produced during the reaction:

$$\begin{array}{cccc} R\text{—}CONH_2 + & OH^- \rightarrow & R\text{—}COO^- + & NH_3 \\ a-x & b-x & x & x \end{array}$$

was determined.

This method is very accurate in the case of slow reactions. For fast reactions, however, it would be expected, that such a procedure is not reliable, as some time is required for the sample to flow through the ion exchanger, and the reaction may proceed to an appreciable extent during this operation. This might under unfavourable circumstances be a serious source of error.

The present investigation was performed in order to study, whether the method would give fairly acceptable values of the velocity constants for fast reactions. In the previous experiments the method was tested against acetamide; in the present investigation cyanoacetamide was used. The hydrolysis of cyanoacetamide proceeds with a velocity, which is about 250 times that of acetamide. Two different methods of analysis of the reaction mixtures were tried:

Method I. The reaction was stopped before the samples were analyzed. For that reason a specially constructed ion exchanger was employed. The ion exchange resin was contained in a one cm wide and seven cm long glass tube, which was fitted with a stopcock at the bottom and a reservoir of about 40 milliliter's volume at the top. Before samples were taken, the stopcock was locked, and a measured quantity of dilute acetic acid was introduced into the reservoir. The sample was then allowed to flow into the acid, when the reaction stopped at once, as the quantity of the acid was enough to make the sample slightly acid. The tap was then opened, and the mixture could flow through the ion exchanger. The sample was then titrated with standardized alkali. In this way the sum of the added acetic acid and the acid from the reaction mixture was obtained.

Method II. The reaction was not stopped before the analysis of samples. The same ion exchangers as in Method I were used. The stopcocks were adjusted in such a manner, that the times for samples to flow through the ion exchangers were the same in all cases. When a sample was taken, it was allowed to flow through the ion exchanger directly. It was assumed, that any correction for the time of this operation would thus be unnecessary.

The measurements showed, that both methods gave corresponding values for the velocity constants.

Methods of calculation

The reaction between cyanoacetamide and alkali is assumed to be of the second order. Thus the following differential equation is valid

$$dx/dt = k(a-x)(b-x) \tag{1}$$

where $(a-x)$ = conc. of amide and $(b-x)$ = conc. of alkali at time t. During the reaction, the quantity x is measured. It is thus most advantageous to calculate k directly from x. This is easily done, when $a=b$. In this case we get on integrating eq. (1)

$$kt = x/a(a-x). \tag{2}$$

Thus

$$ka^2t - katx = x \tag{2a}$$

or

$$x/t = ka^2 - kax. \tag{2b}$$

When x/t is plotted against x, we shall get a straight line with the intercept ka^2 and the slope ka. Eq. (2b) is best treated according to the method of least squares. Then we get

$$a = \frac{\Sigma x^2 \Sigma x/t - \Sigma x \Sigma x^2/t}{\Sigma x \Sigma x/t - n \Sigma x^2/t} \tag{3}$$

and

$$ka = \frac{\Sigma x \Sigma x/t - n \Sigma x^2/t}{n \Sigma x^2 - (\Sigma x)^2}. \tag{4}$$

Eq. (2a) gives often better results than eq. (2b). In the case of eq. (2a) we have x as a function of two variables, t and tx. When eq. (2a) is treated by the method of least squares, we get

$$a = \frac{\Sigma tx \Sigma (tx)^2 - \Sigma tx^2 \Sigma t^2 x}{\Sigma tx \Sigma t^2 x - \Sigma tx^2 \Sigma t^2} \tag{5}$$

and

$$ka = \frac{\Sigma tx \Sigma t^2 x - \Sigma tx^2 \Sigma t^2}{\Sigma t^2 \Sigma (tx)^2 - (\Sigma t^2 x)^2}. \tag{6}$$

Combining eq. (3) and (4) or (5) and (6) we may calculate k without using the initial concentration a.

Another method for the same purpose has been developed by Roseveare (2). In this method, however, it is not possible to take the samples at arbitrary times, as the calculations are based upon a set of three readings, which are taken at equidistant times.

In the present method it is not at all necessary, that the concentrations a and b are exactly equal. As pointed out in a previous paper by the author (3) we can use the integrated equation (2) even when there is a small difference between a and b. It is only necessary to use the mean value $d = \frac{1}{2}(a + b)$ instead of a, so we get

$$kt = x/d(d - x). \tag{7}$$

Thus, on applying eq. (3), (4), (5), and (6), it is sufficient, that a is only approximately equal to b. The quantity "a" from eq. (3) or (5) is then identical with the mean value $d = \frac{1}{2}(a + b)$.

In those cases, where $a = b$, the mean value of k was calculated from eq. (4) or (6). The values of k, which are recorded in the tables, are calculated from eq. (2) by use of the value of a, obtained from eq. (3) or (5). In some experiments the alkali was in excess over the amide. Thus $a \neq b$. Then we get k from the equation:

$$k(b - a)t = \ln[a(b - x)/b(a - x)] \tag{8}$$

which is obtained on integration of eq. (1).

Experimental

The hydrolyses were performed at 25° C, and the temperature was kept constant by a water thermostat with a Vertex-regulator. The cyanoacetamide, "Eastman", was recrystallized three times from alcohol and dried *in vacuo* before use. Samples were taken with a 5-ml pipette. After passing through the ion exchangers, containing Amberlite IR 120, they were titrated with 0.1018-n sodium hydroxide or 0.01917-n barium hydroxide depending on the initial concentrations of the reactants. The indicator was phenolphthalein.

The results of the measurements are shown in Tables I–VII. In all experiments the concentration of alkali was larger than or equal to the concentration of cyanoacetamide. The reaction products are assumed to be sodium cyanoacetate and ammonia. As will be seen from the tables there is good agreement between the velocity constants obtained from each series of measurements.

In the experiments, recorded in Tables I–II, the analyses of the samples were performed according to method I, in which the reaction was arrested by 0.2-n acetic acid. In the other experiments the analyses were performed by method II. In the tables a denotes the initial concentration of cyanoacetamide and b the initial concentration of alkali (NaOH); t is time in minutes.

Table I. $a = 0.0758$; $b = 0.1758$.

t	x	$a - x$	$b - x$	k
5	0.0265	0.0493	0.1493	0.534
10	428	320	1320	576
20	592	166	1166	574
30	670	88	1088	558
40	713	45	1045	576
50	733	25	1025	575
			Mean value	0.566

Table II. $a=b=0.0784$.

t	x	$a-x$	k
5	0.0139	0.0645	0.550
10	240	544	563
20	357	427	533
30	434	350	527
45	519	265	555
60	561	223	535
90	622	162	544
		Mean value 0.541	

Table III. $a=b=0.0847$.

t	x	$a-x$	k
5	0.0171	0.0676	0.596
10	290	557	614
20	418	429	575
30	501	346	570
40	558	289	570
55	615	232	569
75	667	180	584
		Mean value 0.577	

Table IV. $a=b=0.1557$.

t	x	$a-x$	k
5	0.0471	0.1086	0.557
15	0913	644	607
30	1143	414	591
45	1244	314	567
75	1349	208	555
95	1390	167	563
195	1470	87	557
		Mean value 0.568	

Table V. $a=b=0.03434$.

t	x	$a-x$	k
10	0.00528	0.02906	0.529
20	0918	2516	531
30	1220	2214	535
45	1549	1885	535
60	1779	1655	522
90	2127	1307	527
200	2697	737	533
		Mean value 0.530	

Table VI. $a=0.03227$; $b=0.0934$.

t	x	$a-x$	$b-x$	k
5	0.00677	0.02550	0.0867	0.524
10	1235	1992	811	557
15	1614	1613	773	549
20	1939	1288	740	561
25	2180	1047	716	547
35	2494	733	685	547
45	2712	515	663	544
60	2911	316	643	532
			Mean value 0.545	

Table VII. $a=0.01514$; $b=0.0944$.

t	x	$a-x$	$b-x$	k
10	0.00635	0.00879	0.0881	0.598
20	0980	534	846	588
30	1202	312	824	607
45	1381	133	806	637
60	1435	79	801	586
75	1469	45	797	562
90	1488	26	795	545
			Mean value 0.589	

Summary

The velocity of the alkaline hydrolysis of cyanoacetamide has been determined at 25° C. The purpose of the investigation was to determine, whether the ion exchange method for the analysis of reaction mixtures would be useful also for fast reactions. It was found, that acceptable velocity constants were obtained under different reaction conditions.

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On the phase transitions in normal chain carboxylic acids with 12 up to and including 29 carbon atoms between 30° C and the melting point

By EINAR STENHAGEN and ERIK VON SYDOW

With 4 figures in the text

In connexion with investigations of binary systems of normal chain and branched chain carboxylic acids, and in monolayer studies at present in progress, it became necessary to study the crystal behaviour of the normal chain carboxylic acids at different temperatures. Accurate data for the melting and setting points and for the long crystal spacings at room temperature of many members of this important homologous series have been published by FRANCIS and PIPER (1).

The normal chain carboxylic acids are known to possess several crystalline modifications. The even-numbered acids have three forms which have been called A, B, and C, respectively, in order of decreasing long crystal spacing. Four forms, termed A′, B′, C′, and D′, have been reported for the odd-numbered acids (2, 3, 4). The crystalline form found for a certain sample depends on the purity of the sample, on the mode of crystallization, and on the temperature. The information available in the literature with regard to the temperature regions of stability of the various forms is far from complete. DE BOER (5) has investigated the phase transitions for acids with 11, 13, 15, and 17 carbon atoms. He found that the C′ form is stable at higher temperatures and that a reversible transition to the B′ or the A′ form occurs when the temperature is lowered. The data given by DE BOER are included in Table I and it is seen that they agree quite well with our data. DUPRÉ LA TOUR (6) has given corresponding data for the acids with 12, 14, 16, 18, and an "acide cérotique" (supposed to possess 27) carbon atoms. However, this author does not mention anything about the mode of preparation and the purity of the acids used. His results show a very poor agreement with those now obtained, except in the case of the C_{16} acid where his value for the B→C transition falls on the extension of our curve (Fig. 1). No data have been found for acids with more than 18 carbon atoms.

We have studied the stability ranges of the different modifications of the normal chain acids with 12 up to and including 29 carbon atoms with the aid of an X-ray camera designed for continuous recording of diffraction pattern-temperature (DPT) diagrams (7).

Material used

For an investigation of this kind it is of course essential to use fatty acids of the highest degree of purity possible. All acids except C_{28} have been synthesized in the

Table I a

Number of carbon atoms	Melting point °C		Long spacings Å						Transition point on heating °C		
			A		B		C				
	Present investigation from DPT diagram	Literature a)	Present investigation	Literature	Present investigation	Literature	Present investigation	Literature	A→C	B→C	Literature
12	44.0	44.8 b)	32.1				27.4	27.42 b) 27.31 c)	32		
14	54.2	54.4	36.7	36.64 c)			31.7	31.60 a)	44		
16	62.9	62.9	41.7	41.2 d) 41.0 f)		39.1 a)	36.0	35.60 a)	59		40 e) (B→C)
18	69.7	69.6	46.5	46.29 c) 46.6 f)	44.1	43.75 a)	40.0	39.75 a)	54	46	
20	75.1	74.35			48.8	48.45 a)	43.9	44.15 a)		52	
22	79.9	79.95	55.5			52.95 a)	48.3	48.3 a)	63		
24	83.9	84.15			57.8	57.75 a)	52.9	52.6 a)		65	
26	87.5	87.7			62.2	62.2 a)	57.1	56.25 a)		72	
28	90.7	90.9			67.5	67.15 a)	61.6	61.05 a)		79	

a) FRANCIS, PIPER (1)
b) VAND *et al.* (14)
c) SLAGLE, OTT (4)
d) DE BOER (5)
e) DUPRÉ LA TOUR (6)
f) FRANCIS, PIPER, MALKIN (3)

Table I b

Number of carbon atoms	Melting point °C		Long spacings Å						Transition point on heating °C			
	Present investigation from DPT-diagram	Literature	A′		B′		C′		A′→B′	A′→C′	B′→C′	Literature
			Present investigation	Literature	Present investigation	Literature	Present investigation	Literature				
13	41.4	41.55 c)	35.1	34.92 c) 35.3 f)			29.6	29.8 c) 30.0 f)		34		32 ($\beta_1-\alpha$) e) h)
15	52.1	52.26 c)	40.2	39.88 c) 40.0 f)	36.4	35.75 c)	34.5	34.4 e) 34.2 f)	45		46	44 ($\beta_{11}-\alpha$) e) h)
17	61.1	61.3 a)			40.3	40.45 a)	38.5	38.57 c) 38.7 e) 38.6 f)			56	54 ($\beta_{11}-\alpha$) e) h)
19	68.5	68.65 a)	49.2		44.7	44.50 a)	43.0	43.15 f)	52		66	
21	74.8	74.3 a)			49.1	49.25 a)	46.9	47.8 f)			72	
23	79.1	79.1 a)			53.6	53.40 a)	51.0				77.5	
25	83.2	83.5 a)			58.0	57.65 a)	56.2				81.5	
27	87.5	86.8–87.0 g)			62.7		60.0				85	
29	90.4	90.3 a)			66.6	66.35 a)	65.2				89	

g) S. Shiina, J. Soc. Chem. Ind. Japan, Supplemental Binding *39*, 180 B (1936)

h) In de Boer's nomenclature β_1 corresponds to the A′, β_{11} to the B′, and α to the C′ form.

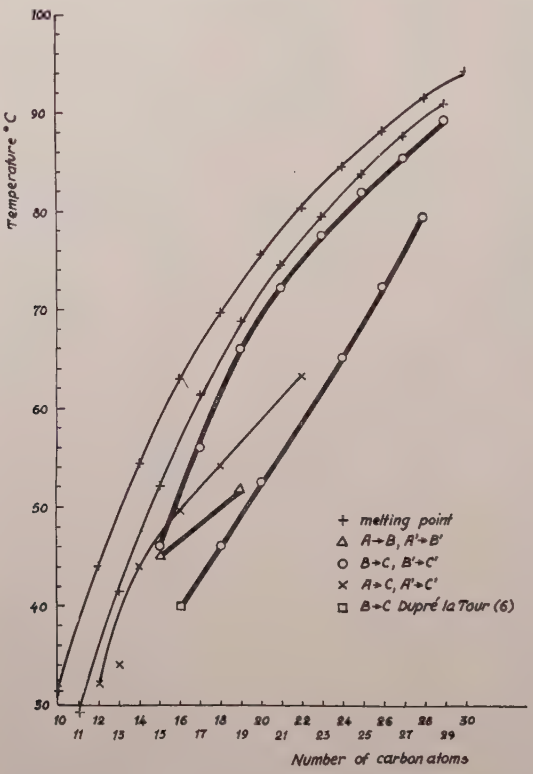

Fig. 1. Melting and transition points for *n*-fatty acids.

Institute of Medical Chemistry, and have been carefully purified until melting and setting points agreed with those given by FRANCIS and PIPER (1). A set of samples of the acids described by these authors had been obtained from the late Professor F. FRANCIS in 1939 and were used for comparison, except in the case of the C_{12}, C_{13}, C_{15}, C_{27} acids, which were not described by FRANCIS and PIPER. Melting points and crystal spacings are given in Tables I a and I b.

Several of the specimens prepared in Uppsala have been described in previous communications. The C_{12} up to and including the C_{20} acids have been described by STÄLLBERG-STENHAGEN (8), and the C_{22} acid by STÄLLBERG-STENHAGEN and STENHAGEN (9). The C_{21} acid was prepared via the methyl ester obatined by mixed electrolysis of methyl hydrogen dodecane-1,12-dicarboxylate and *n*-nonanoic acid. The ester had melting point 47.2°, setting point 46.4°, and re-solidification point 46.6–46.8°. FRANCIS and PIPER (1) give m.p. 47.2° (β-form), r.p. 46.6°. The free acid was distilled in vacuo and finally crystallized from light petroleum (b.p. 40–60°). The C_{23} and C_{24} acids were synthesized from *n*-docosanol-1 via the iodide and nitrile and malonic ester synthesis respectively. The C_{25} acid was prepared by CLEMMENSEN reduction of 13-ketopentacosanic acid of m.p. 96.2–96.5°, and the C_{26} acid by the same method from 13-ketohexacosanoic acid of m.p. 98.1–98.3°. The C_{27} acid was obtained by reduction of 13-ketoheptacosanoic acid of m.p. 100.2–100.4° using

Table II

Crystal form at a temperature of 30° for acids crystallized under different conditions

	Crystallized from		
	Glacial acetic acid	Light petroleum	Melt
Even numbered acids			
12	C	A	C
14	C	A	C
16	C	A	C
18	A + C	B + C	C
20	B + C	B + C	C
22	–	A + C [a])	C
24	–	B + C	C
26	–	B + C	C
28	–	B	C
Odd numbered acids			
13	A′	A′	A′
15	A′ + B′	A′	B′
17	B′	B′	B′
19	B′	B′	B′
21	–	B′	B′
23	–	B′	B′
25	–	B′	B′
27	–	B′	B′
29	–	B′	B′

[a]) Crystallized from benzene.

Huang-Minlon's modification of the Wolff-Kishner method. The procedures used have been described previously (10, 11). The C_{29} acid has been described by Ställberg, Ställberg-Stenhagen, and Stenhagen (12).

X-ray studies

The X-ray investigations were performed with specimens deposited on small glass plates (cf. ref. (1)). Nickel-filtered copper K-radiation was used (Philips-Müller tube). The rate of heating (or cooling) of the specimen in the recording of the diffraction pattern-temperature (DPT) diagrams was 15° per hour. The specimens were rocked over an angle of 10°, and the distance between the specimen in the thermostat and the film was 10 cm.

The specimens used were obtained by crystallization from solvents or from the melt. It is well known (cf. (13)) that the crystal form adopted in a certain case often depends on the nature of the solvent used for the crystallization. In the present case we have used light petroleum (b.p. 40–60° for acids containing less than 20 carbon atoms, and b.p. 95° for the higher acids), and glacial acetic acid. Our results are given in Table III. According to Piper, Chibnall, and Williams (13), the co-existence of two crystalline forms in an acid crystallized from a solvent is an

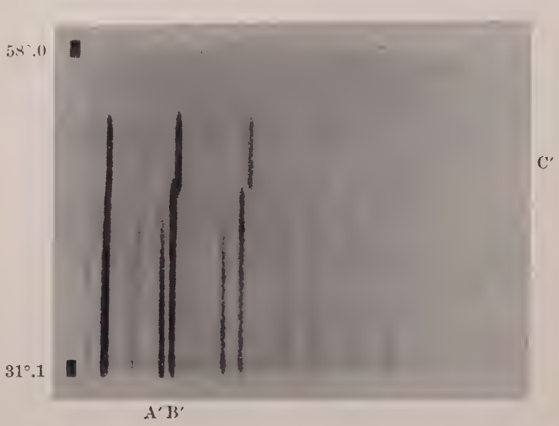

Fig. 2. Diffraction pattern-temperature diagram for *n*-pentadecanoic acid crystallized from glacial acetic acid.

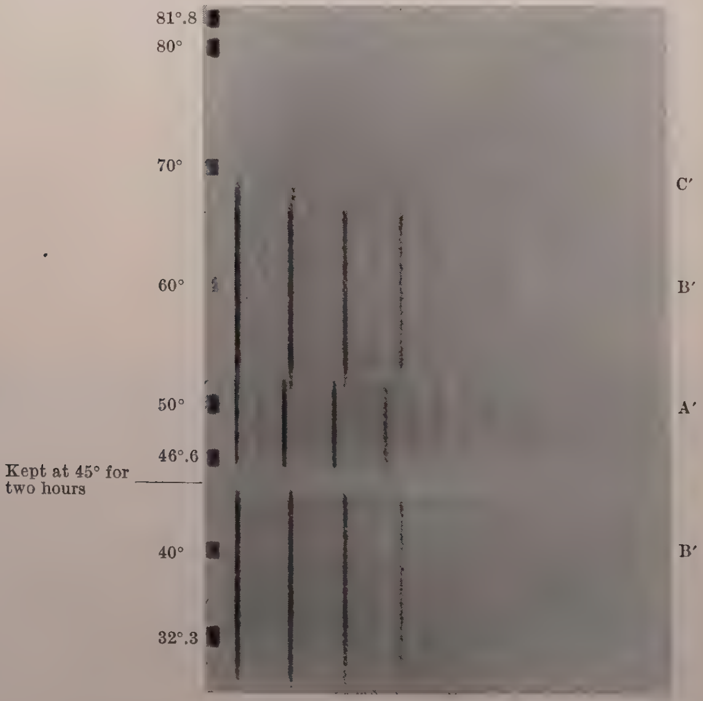

Fig. 3. Diffraction pattern-temperature diagram for *n*-nonadecanoic acid crystallized from acetic acid.

indication that the specimen is impure. However, the results of Piper *et al.* were obtained with acetone as solvent, and Francis and Piper (1) have later found that pure specimens of the C_{20} acid gives a mixture of the B and C forms when crystallized from glacial acetic acid. As shown in Table III this was also found in the present case. It is evident that in particular crystallization from light petroleum of even-numbered acids often gives a mixture of two crystalline forms even when the specimens used are pure (cf. also (3)).

In agreement with the findings of FRANCIS, PIPER, and MALKIN (3), we obtained in the case of even-numbered acids the C form on slow cooling of the melt, whereas the odd-numbered acids under these conditions are obtained in the B′ form at room temperature, the only exception being the C_{13} acid which shows the A′ form. It is also in agreement with the findings of FRANCIS and PIPER (1) that the odd-numbered acids with more than 15 carbon atoms are obtained in the B′ form when crystallized from solution.

The results are collected in Tables I a and I b, Table II, and in Fig. 1.

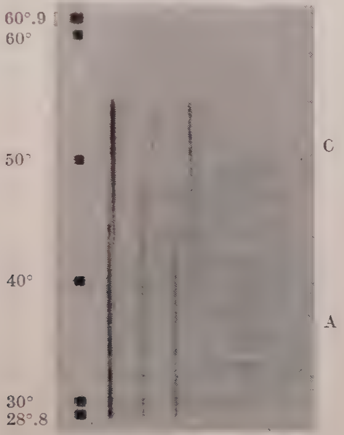

Fig. 4. Diffraction pattern-temperature diagram for *n*-tetradecanoic acid, showing persistence of the second order line in the transition A→C.

In the course of the present investigation we have observed the A form for the C_{12} and C_{22} acid and the A′ form for the C_{19} acid. The C_{27} acid does not appear to have been studied previously, but as expected it shows the B′ form at lower temperatures, changing to C′ near the melting point. Values for the C′ spacing of the C_{23}, C_{25}, C_{27}, and C_{29} acids have not been published before. The D′ form of the odd-numbered acids has not been observed in the course of the present work. The information about this crystal form in the literature (3, 4) is very scanty.

The long crystal spacings $(0\,0\,l)$ usually increase with the temperature, the coefficient of expansion being 0 – 0.02 Å per degree C, the values being fairly constant for each crystal form.

It is known from the work of GARNER *et al.* (15, 16), and DE BOER (5), that the lower odd-numbered acids starting with *n*-nonanoic acid (15) show a reversible phase transition some degrees below the melting point. As shown in Fig. 1 all odd-numbered acids with 15 or more carbon atoms show a reversible transition $B' \rightleftarrows C'$. The C_{13} acid shows a reversible transition $A' \rightleftarrows C'$, whereas the C_{15} and the C_{19} acids when obtained in the A′-form on heating first undergo a transition $A' \rightarrow B'$. A DPT diagram for the C_{15} acid is reproduced in Fig. 2. It was found that a specimen of the C_{19} acid, originally obtained in the B′ form by crystallization from glacial acetic acid, when kept at a temperature of 45° underwent a transition into the A′ form (Fig. 3).

For the even-numbered acids, C appears to be the stable form, as the transitions A → C and B → C are not reversible. A transition A ⇄ B has not been observed.

The reversible transitions are quite sharp, but show hysteresis, in that they occur at higher temperatures on heating than on cooling (cf. GARNER and RANDALL (15)). In order to determine the exact equilibrium temperature an extremely slow rate of change in the temperature must be employed. When the C_{13} acid was cooled at a rate of 15° an hour the transition occurred at 26°. Under similar conditions the C′ → B′ transition of the C_{15} acid took place at 43°.

The irreversible transitions often take place over a temperature interval of several degrees when the specimen is heated at a rate of 15° an hour (cf. Fig. 2). In transitions of this type it is often observed that the diffraction lines of even order persist to much higher temperatures than those of odd order, in spite of the fact that the odd order reflections are initially the stronger ones. This means an effective halving of the long crystal spacing during the transition. The appearance of the A → C transition in the case of C_{14} acid is shown in Fig. 4.

Acknowledgements

We are indebted to Mrs. STINA STÄLLBERG-STENHAGEN, Miss GUNNEL STÄLLBERG, and Mr. KLAUS SERCK-HANSSEN for material used, and to professor GUNNAR HÄGG for valuable discussions. The expenses involved have partly been defrayed by grants from *Statens naturvetenskapliga forskningsråd.*

SUMMARY

A study of the phase transitions in the normal chain carboxylic acids with 12 up to and including 29 carbon atoms over the temperature range 30°-melting point has been made, using an X-ray camera that records the diffraction patterns as a function of temperature. Transition temperatures have been determined and in the case of the A, A′ and C′ forms some new crystal spacing data have been recorded.

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Experiments with microseparation of liquids (solvents) with displacement adsorption on silica gel

By SVEN-GÖSTA BLOHM

With 28 figures in the text

At the first international microchemical congress the main principles of a technique for the microanalysis of fluids by means of adsorption was presented (1). The technique is to be regarded as a micro-performance of B. J. MAIR's method (2), which has latterly been made the object of comprehensive investigations, above all at the American petroleum laboratories (3), in which connection important and fruitful results have been obtained as regards the analysis of fluids belonging to the class of petroleums.

The aim of the present paper is in the first place to communicate supplementary information concerning the microtechnique, which has in certain details been modified and simplified, and in the second place to present experimental results intended to illustrate the usefulness of the method in examinations of fluids belonging to the class of solvents (hydrocarbons, chlorinated hydrocarbons, acetates, terpenes). As total an analysis as possible of such fluid mixtures is important from, inter alia, the industrial hygienic viewpoint (4), and experience has shown that there is a great need for supplementary methods of analysis in this field. This is connected with, amongst other things, the copius occurrence of azeotropes in such mixtures of solvents (5), with the great number of components and with the minimal quantities of fluid to be analysed. Fractional distillation at this laboratory is performed on a semi-microscale (6), the fraction-volumes being parts of the milliliter, and these fractions are particularly interesting to investigate further. This fact, together with the considerable saving of time and materials, constitutes the chief reason for the use of the microscale. According to the present modified technique the time required for an orienting examination of a fluid is about 1/4 hour, and the amount of fluid required about one drop.

To be able to interpret the result of a fluid analysis by means of displacement adsorption, one needs in the first place to know the adsorption sequence of the different components, i.e. their mutual affinities to the adsorbent used. For hydrocarbons (7) and sulfurated hydrocarbons (8) such a sequence series has, in its main features, been drawn up.

As regards the adsorption affinity for fluids belonging to the class of solvents, several investigations have been carried out, where the problem has been treated from the viewpoint of a search for fluids or fluid mixtures with well-balanced eluating capacity for the separation of higher molecular component mixtures. The most comprehensive work has been performed by W. TRAPPE (9) in the presentation of his series of eluotropic solvents. Other presentations have been made by e.g. STRAIN (10) and HESSE (11).

No work directly intended to illustrate the mutual adsorption sequence for solvents and the conclusions that may be drawn therefrom concerning the possibilities of separation by means of displacement adsorption seems to have been performed. The author has therefore tried to obtain an orientation in this field, and a sequence-series comprising about 20 solvent components has been drawn up.

Apparatus and technique

For the account of the apparatus as such, the reader is referred to the earlier paper (1). Here I will only touch upon the modifications that have been made.

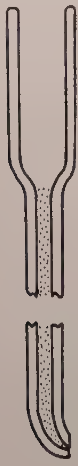


Fig. 1. The micro column.

The column, Fig. 1, consists of a glass tube of even thickness whose lower part has been drawn out to a fine opening which is at right angles to the main direction of the column. The region nearest this lower opening is ground so as to produce a round plane surface with a diameter of about 1 mm. The opening should be so fine, about 5/100 mm, that the adsorbent is retained quantitatively, while the fluid can pass freely. This detail gives a greater simplicity as compared with the system of glass ball and notched tongue (reference 1, Fig. 3).

Suitable dimensions for the capillary tubes in which the fractions are collected (1, Fig. 4) have been found to be: length 150 mm, outer diameter 3 mm, inner diameter 0.6 mm, angle of bend 170°. In order to obtain a more exact measurement of the fractions, the capillaries have been graduated in cu.mm. Further, the upper part of the capillary rack has been blackened, in order to facilitate the reading of the front of the column of fluid. It is important that the capillaries should be exchanged with great speed and precision, in order, amongst other things, to diminish the error due to evaporation and increase the reproduceability. This is made easier if the capillary rack rests against a metal strip along which it may be pushed horizontally.

Identification of the fractions

In order to obtain an indication of the composition of the fractions, volume 5 cu.mm, the micro-refractometer technique worked out by the author (12) has been used throughout. Another method that has been tried has been the measurement of specific gravity, the capillaries here serving as the pyknometer. The accuracy of such a measurement of specific gravity is ± 0.1–0.05 g/ml, and it may serve as an orientation in the separation of samples containing chlorinated hydrocarbons. For a qualitative indication of these, a modification

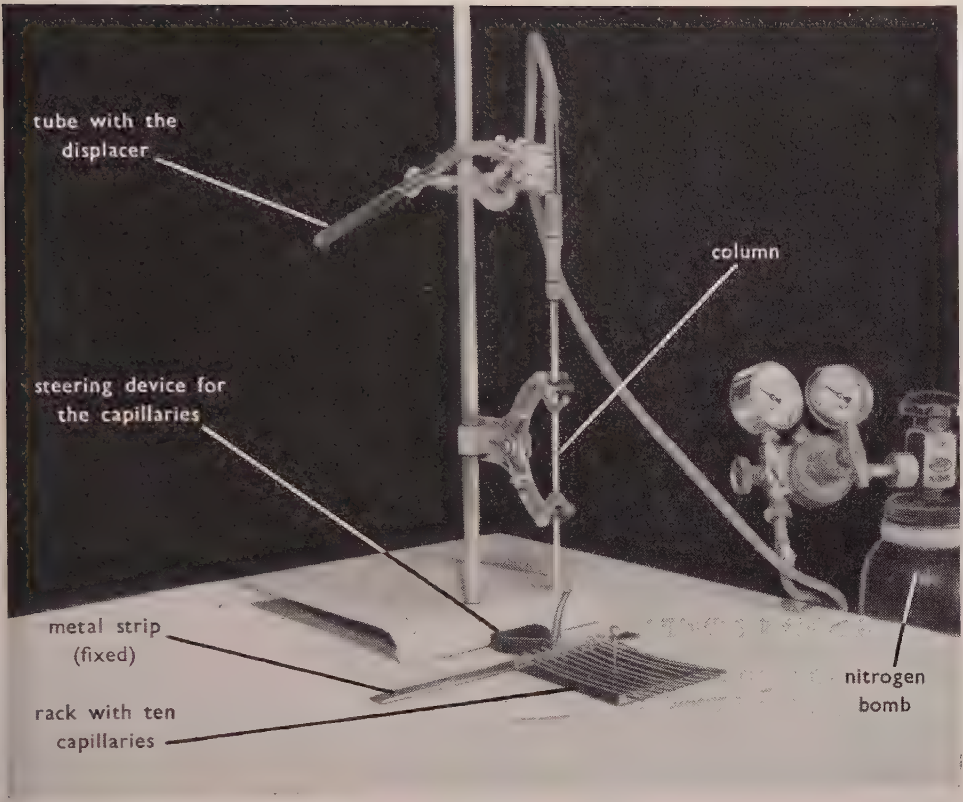


Fig. 2. Apparatus for microanalysis of liquids by displacement adsorption.

of the Beilstein test has been worked out. This is based upon the same idea as the technique communicated by Ruigh (13), i.e. one mixes coal-gas with the sample, whereupon the gas is ignited and is allowed to meet a copper wire. Procedure: the copper wire, which is attached to the micro burner (Fig. 3), is warmed up with a bunsen burner. The gas supply-pipe to the micro burner is made to pass through the capillaries in which the fractions have been collected. The test is so sensitive—a distinct flame-reaction is obtained for a quantity of chlorinated hydrocarbon of about 1/100 mg (measured for CCl_4)—that the traces of fluid remaining in the capillaries after their contents have been used for e.g. the measurement of refraction index are sufficient to show the presence of chlorinated hydrocarbon.

Adsorbent

Silica gel has been used throughout as surface-active substance. The gel has been activated in a drying pistol according to Abderhalden—entirely of glass—with xylol as boiling fluid for 4–5 hours. The activation was performed in vacuum, in order that the moisture in nonactive gel may be removed

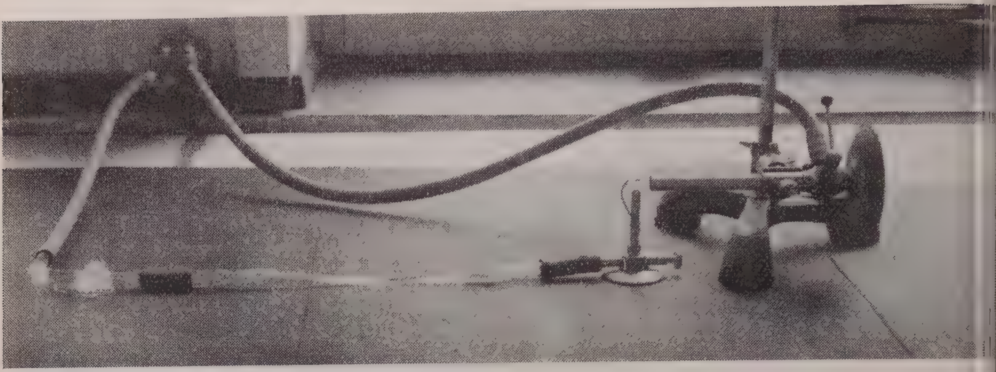


Fig. 3. Arrangement for qualitative halogendetermination in the capillaries.

smoothly. In order to get the adsorbent homogeneously packed in the extremely narrow columns that must be used for the micro-scale, the packing has been done with vibration, the lower part of the column being made to rest against the vibrator. Experiments with different amplitudes (at 100 P/s) showed that maximum packing was already obtained at 1/10 mm amplitude after a minute or so. With greater amplitudes the gel began to "boil" in the column, which entailed a poorer packing effect as well as increased risk of breakage for the column.

Removal from the column of adsorbents used was effected with the technique of heat-vacuum (1, Fig. 7). For smaller columns a careful warming with open flame in combination with vacuum is quite sufficient. The temperature at which the moistened gel leaves the column is with ethyl alcohol as displacer about 70° C.

Losses due to evaporation

The phases during which losses due to evaporation may arise are firstly at the transference of fluid from column to capillary and secondly while the fluid is in the capillaries. The losses during the last-mentioned phase were tested for 10 cu.mm n-pentane introduced up to the middle of a capillary, which was then weighed at regular intervals. They were found to be entirely negligible—no noticeable loss, greater than 1 %, could be observed until after 1/4 hour—as compared with the considerable losses which were observed during the first-mentioned phase. These losses have been tried out by displacing a known amount of different pure fluids and determining from the index of the fractions the collected amount of the pure fluid. In experiments with 40 cu.mm sample collected in 10 fractions of 5 cu.mm (the last fraction contains only the displacing liquid, absolute ethanol) and a percolating rate of 5 cm/minute for columns with a diameter of 1.4 mm, the loss for e.g. *n*-pentane (boiling-point 36° C) was about 20 %, $CHCl=CHCl$, trans (boiling-point 49° C) about 10 %, $CHCl_2CHCl_2$ (boiling-point 146° C) about 5 %.

Range of usefulness

Sequence of solvents

As has already been mentioned, displacement adsorption has as far as I know not been used for the solution of other problems of fluid analysis than those arising in the petroleum industry. To get some idea of the extent to which this technique of separation may be useful for the analysis of fluids belonging to the class of solvents, a series of experiments have been performed, with the aim of ascertaining the sequence in which the fluids may be arranged according to their mutual affinity to silica gel.

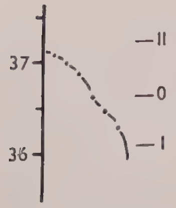
The fluids that have been tested in this way have been some hydrocarbons, chlorinated hydrocarbons and simpler acetates.

The method employed has been of a purely empirical character, and has consisted in testing successively against each other different binary fluid mixtures (conc. about 50–50 volume-percent measured as a mean value of the refraction-index of the components). The apparatus used is: column inner $\phi = 1.40$ mm, length 250 mm; adsorbent Silica Gel, The Davison Chemical Corporation (22–08–X1926), through 200 mesh; amount of adsorbent corresponding to 250 mm filling, 0.35 grams; displacer absolute ethanol (for methyl acetate, methanol, absolute, has been used); added amount of sample about 40 cu.mm and fraction volume 5 cu.mm (Fig. 2). A suitable rate of percolation was obtained by excess pressure from a N_2-bomb, 1.2 kg/cm^2. The tests were performed at room temperature.

From the form of the adsorptograms (index against volume) obtained it has been possible to determine the sequence of the components entering in the sample; from the shape of the curves an estimation of the selectivity has also been possible. This lastmentioned estimation has in a high degree facilitated the discovery of the sequence that the fluids finally proved to have in relation to one another. Owing to the great differences in boiling-point and therewith approximatively also in the volatility shown by some pairs of fluids lying close to each other in the series (see Table I)—e.g. $CCl_2 = CCl_2$/*n*-pentane and $CH_2Cl_2/CH_2Cl\ CHCl_2$—and the source of error, entailed in the technique itself, constituted by the error due to evaporation, it is only possible to attach semi-quantitative value to the measurements.

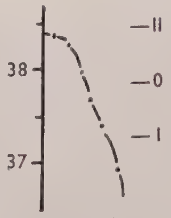
With such a successive testing of fluids in relation to each other, under the conditions described above, it proved that for each fluid was obtained a definitive placing in relation to the other fluids, and it has not been possible to observe any reversal of sequence. (See also the "Discussion", p. 8.) That is to say, if the fluids A and B had the sequence A > B and a third fluid C was tested against B and found to have a lower affinity, B > C, then one found also A > C, as was also to be expected. One could thus conceivably obtain the sequence by testing all the components against one fluid as standard (14, 15). The experiments I have carried out with this aim, i.e. to shake a binary mixture with a known amount of gel and measure the difference in concentration before and after, showed, however, that with the available metrical resources the uncertainty of such a determination was all too great.

The preliminary placing of the fluids in the series, see table I, has been done partly by the method of trial and error, but a certain guidance has been obtained from e.g. TRAPPE's series (l.c.).



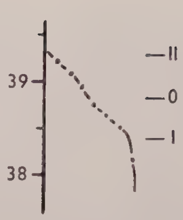
methylac I
ethylac II

Fig. 4



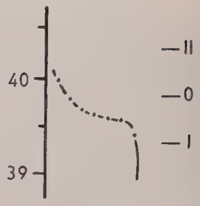
ethylac I
n-propylac II

Fig. 5



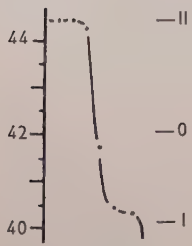
n-propylac I
n-butylac II

Fig. 6



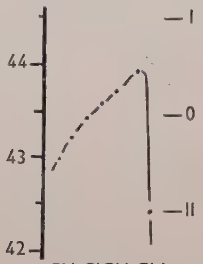
n-butylac I
n-amylac II

Fig. 7



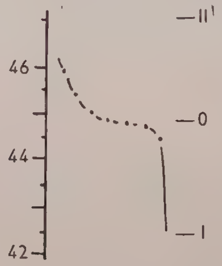
n-amylac I
CH_2ClCH_2Cl II

Fig. 8



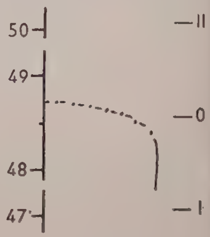
CH_2ClCH_2Cl I
CH_2Cl_2 II

Fig. 9



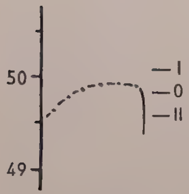
CH_2Cl_2 I
$CH_2ClCHCl_2$ II

Fig. 10



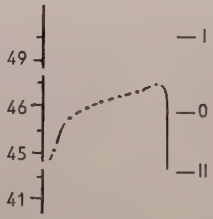
$CH_2ClCHCl_2$ I
benzene II

Fig. 11



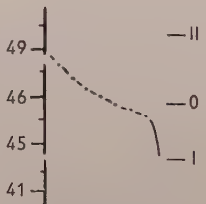
benzene I
toluene II

Fig. 12



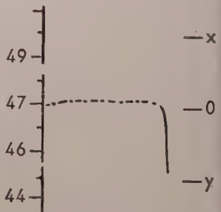
toulene I
CH_3CHCl_2 II

Fig. 13



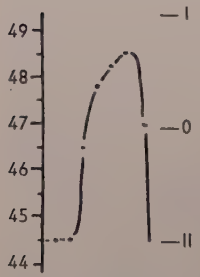
CH_3CHCl_2 I
$CHCl_2CHCl_2$ II

Fig. 14



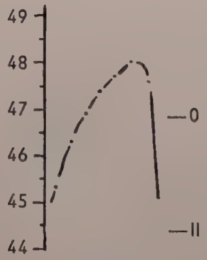
$CHCl_2CHCl_2$ x
$CHCl{=}CHCl$, cis y

Fig. 15



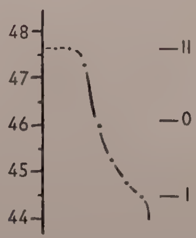
$CHCl_2CHCl_2$ I
$CHCl{=}CHCl$, trans II

Fig. 16



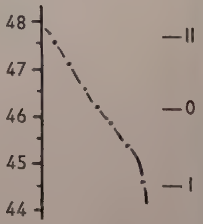
$CHCl_2CHCl_2$ I
$CHCl_3$ II

Fig. 17



$CHCl_3$ I
$CHCl{=}CCl_2$ II

Fig. 18



$CHCl{=}CHCl$, trans I
$CHCl{=}CCl_2$ II

Fig. 19

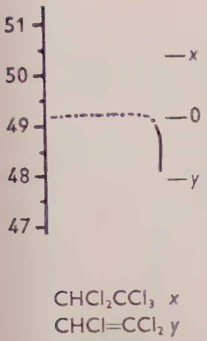


Fig. 20

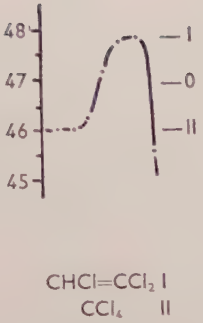


Fig. 21

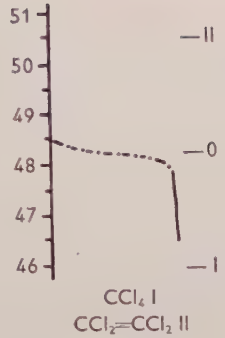


Fig. 22

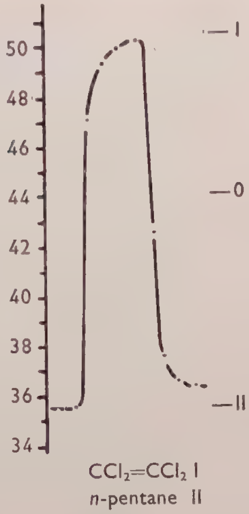


Fig. 23

Figs. 4–23. Adsorptograms showing the mutual adsorption affinities towards silica gel for some solvents compounds in concentration 1 : 1.

In Figs. 4–23 are shown the adsorptograms that have been obtained between pairs of fluids lying close to each other in the sequence. The refractive indices as a direct function of $n^{20}D$–1/100 are plotted on the ordinates, which for the sake of clarity have been given varying index-intervals. All the adsorptograms have the same abscissa (excluded in the figures) representing fraction volumes. Each point connotes index and corresponding volumetric value for 5 cu.mm fractions. I and II connote the indices for the strongest and the weakest adsorbed components respectively. 0 is the index of the mixture. In the majority of the adsorptograms only that part of the curve which illustrates the separation of the sample itself has been reproduced, as the transition between sample and displacer—which with the apparatus-dimensions used has been 2–3 fractions—has for all the systems a very similar appearance, i.e. it goes rapidly downwards towards the vicinity of the index for the displacer, to which it then gets successively nearer (see Fig. 23). It is a matter of primary interest to establish that all the systems treated here are completely displaceable by the lower alcohols.

The accuracy of the index-measurements is ± 0.0001, of the measurements of fraction volumes approximately ± 0.2 cu.mm and for the put in of the sample, which was performed with a syring operated micro-voll-pipette which had been thoroughly drained, the accuracy was approximately ± 1 cu.mm. It is thus possible, with in other respects similar experimental conditions, particularly in the collection of the fractions (see "Losses due to evaporation", p. 4), to obtain good reproduceability.

All the components have been purified through a distillation column with 25 theoretical plates (6). The boiling-point obtained for the middle fraction which was selected is given in Table I. Each of the components has also been subjected to an individual adsorption analysis on the same scale as in the

Table I

The fluids arranged according to falling affinity to silica gel. The sequence obtained by successive testing of binary mixtures (1 : 1) against each other. Figs. 4–23.

Fig.	Liquid	BP °C[1]	$n_D^{20}-1$[2]
4	CH_3COOCH_3	56.7	3611
4, 5	$CH_3COOCH_2CH_3$	77.1	3726
5, 6	$CH_3COO(CH_2)_2CH_3$	101.8	3847
6, 7	$CH_3COO(CH_2)_3CH_3$	124.4	3930
7, 8	$CH_3COO(CH_2)_4CH_3$	146.7	4028
8, 9	CH_2ClCH_2Cl	82.0–82.5	4448
9, 10	$CH_2\ Cl_2$	39.5	4241
10, 11	$CH_2Cl\ CHCl_2$	113.4	4709
11, 12	C_6H_6	80.1	5011
12, 13	$C_6H_5 \cdot CH_3$	109.8	4961
13, 14	$CH_3\ CHCl_2$	57.1	4169
14–17/15	$CHCl_2CHCl_2/CHCl=CHCl$, cis	146.5/60.6	4939/4440
17, 18	$CHCl_3$	60.9	4459
16, 19	$CHCl=CHCl$, trans	48.7–49.0	4449
19–21/20	$CHCl=CCl_2/CHCl_2CCl_3$	86.0/159.5	4773/5030
21, 22	CCl_4	77.0	4600
22, 23	$CCl_2=CCl_2$	120.0–120.5	5051
23	C_5H_{12}	36.0	3571

[1] Transferred from mV. The distillation apparatus (6) was provided with thermoelement. The values are not corrected for variations in the atmospheric pressure.

[2] Meanvalues from adsorptograms from the purified liquids. Maximum deviation in the sample level ±0,0002. Temperature constancies from ultra-thermostat, Hoeppler.

sequence experiments. It was in this connection not possible to observe any adsorption-cracking recordable with refraction index. A maximum deviation of ± 0.0002 index-units in the testing level of the adsorptogram has in this connection been tolerated. For some liquids the distillation had to be completed with other methods in order to obtain this criterion (see Indication of inhomogenieties, p. 10). The mean values for the refraction indices thus obtained are included in Table I.

Discussion of the sequence series

For homologous series (see Table I) the affinity decreases with rising molecular weight, e.g. the chlorinated methanes, acetates, and for the chlorinated ethanes and ethenes with increasing content of chlorine. The ethene chlorinated hydrocarbons have throughout a lower affinity than the corresponding ethane derivatives; e.g.: CH_2ClCH_2Cl is more strongly adsorbed than cis- and trans $CHCl = CHCl$, and $CHCl_2CHCl_2$ more strongly than $CCl_2 = CCl_2$. This is the contrary of what was found for the hydrocarbons, where olefins are throughout more strongly adsorbed than aliphatic compounds (2). In the separation of hydrocarbons it has also been found (2) that a clear group-division exists, e.g. aromatics > olefins > aliphatics. Also for solvents it is possible to distinguish a certain division into groups. According to Mair's and co-workers' investigations (7) the affinity for the aliphatic hydrocarbons increases with decreasing molecular weight. The most strongly adsorbing fluid in this group will therefore be pentane. The position of this fluid in the sequence series and the sharp separation obtained with the next nearest fluid, $CCl_2 = CCl_2$ (Fig. 23) make it possible to assume that all the aliphatic hydrocarbons are easy to separate from every other component in the series. Another group-division is obtained through a comparison with the position of the acetates and the other fluids. The sharp separation obtained for *n*-amyl acetate CH_2ClCH_2Cl (Fig. 8) indicates that every other component in the series under the acetates is easy to separate from these.

There does not seem to be any marked group-division of the different chlorinated hydrocarbons and the aromatics; these fluids are infiltrated in each other from adsorption affinity. In certain cases it has been observed that the degree of separation has been very weak, e.g. $CH_2ClCHCl_2$/benzene (Fig. 11), benzene/toluene (Fig. 12), toluene CH_3CHCl_2 (Fig. 13). For the systems $CHCl_2CHCl_2/CHCl = CHCl$, cis (Fig. 15) and $CHCl = CCl_2/CHCl_2CCl_3$ (Fig. 20) the same affinity was obtained at the given concentration, i.e. no noticeable separation. In the determination of the sequence for $CHCl = CHCl$, trans and $CHCl_3$, which are not directly comparable with each other owing to the fact that their refractive indices are all too close, it has been found that both of these fluids have a lower affinity than $CHCl_2CHCl_2$ (and therewith also $CHCl = CHCl$, cis), Figs. 16 and 17, and higher affinity than $CHCl = CCl_2$ (and therewith also $CHCl_2CCl_3$), Figs. 18 and 19. From the form of these adsorptograms it appears that $CHCl_3$ has a stronger affinity than $CHCl = CHCl$, trans. From Figs. 15 and 16 it emerges also that cis- and trans $CHCl = CHCl$ are separable by adsorption. Cf. HESSE and TSCHACHOTIN (16).

This sequence series illustrates conditions that are valid at the concentration 1:1. A factor that complicates the analysis of fluids by means of adsorption is the circumstance that the affinity for the fluids relatively to one another is in certain cases dependent upon concentration and that reversals of sequence may take place. This applies especially for difficultly separable systems, and for such systems only the most weakly adsorbed component can be obtained in the pure form. For a detailed theoretical discussion of these questions the reader is referred to HIRSCHLER and AMON's work (17).

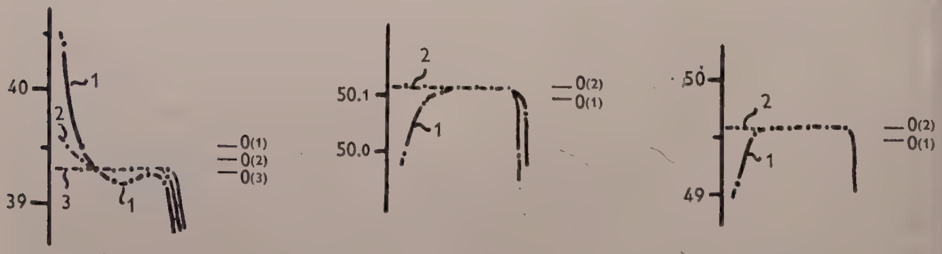
It is therefore possible to obtain a total picture of the sequence conditions only if all the fluid systems are investigated over the entire range of concentrations—an investigation which falls, however, outside the scope of this work. In comparison with TRAPPE's series (l.c.) one finds on the whole good agreement. A marked exception, however, is the position for $CHCl_3$ which TRAPPE found to have higher affinity than CH_2Cl_2. Such a sequence diverges, however, from the rule that for homologous series the affinity decreases with rising molecular weight.

From the practical point of view it is of interest to observe that no connection between adsorption affinity and boiling-point for the fluids appears on the whole to exist (see Table I). This circumstance reduces the probability that fluid mixtures are difficultly separable as well by means of distillation as by adsorption. This increases the complementary value of the methods. It also directly emerges from the experiments that all the azeotropes formed by the lower alcohols with the other components (5) are very easy to separate by means of this analytical technique.

Indication of inhomogeneities

The apparatus-dimensions which have been used to obtain the sequence-series are of course not well-adapted for a more total fractióning of a fluid sample as the sample volume employed permits one to obtain only a limited number of fractions. Only for very easily separable systems, e.g. Figs. 5, 6, 8, 16, 18, 21, 23, a component can be isolated. The practical advantage of the micro-method in the analysis of unknown fluid mixtures is, however, that one is enabled to decide rapidly and with a minimal consumption of material whether displacement adsorption is a practicable method for further analysis, and if so, to carry out a more complete examination on a bigger scale. If the amount of the sample is increased, in order to obtain a greater number of fractions, both the ratio of the gel-amount to the sample and the ratio of the length of the sample to the diameter of the column should be as great as possible. In the first place one should therefore, with unchanged diameter, increase the amount of adsorbent proportionally to the amount of the sample. The rate of percolation diminishes greatly, however, with increasing length of column at constant diameter, whereby the error due to evaporation increases. In this way one soon reaches an upper limit for the possibility of compensating the increased amount of sample simply by increasing the length of the filling. One must thus also have recourse to an increase in the diameter of the column when working with larger amounts of sample. For a detailed discussion of the connection between separation-effect, amount of sample and apparatus-dimensions the reader is referred to HIRSCHLER and AMON (17) and FINK and co-workers (18), of whom the last-mentioned have since quantitatively determined these variables in order to obtain an optimal separation of aromatic–aliphatic hydrocarbon mixtures.

The fluids that have been used in the sequence investigations have, as has already been mentioned, been tested in respect of their purity on the same scale. Examples of the marked indications of inhomogeneity obtained in this connection for some of the fluids examined before the purification are shown in the Figs. 24–26.



n-butylacetate

Fig. 24

benzene

Fig. 25

toluene

Fig. 26

Figs. 24–26. Indication of inhomogeneities.

Fig. 24. *n*-Butyl acetate 1. "Eastman Kodak." 2. (1) distilled, BP 125.6° C. 3. Own preparation.

Fig. 25. Benzene. 1. "Baker's Analyzed." 2. (1) adsorption purified.

Fig. 26. Toluene. 1. "Baker's Analyzed." 2. (1) adsorption purified.

From commercial *n*-butyl acetate it was not possible to obtain a smooth level in spite of careful distillation or purification by adsorption. This liquid, as well as the other acetates, was therefore prepared from highly purified acetylchlorid and the corresponding alcohols.

One advantage in using the method for determinations of purity is that no absolute index-values are necessary, as the method is based upon the recording of differences in index. As the readings are made at very short intervals, even a refractometer with a defective or arbitrarily chosen temperature-constancy may be used. An even index-level is, however, not an unequivocal proof that the fluid examined is homogeneous; see Figs. 15, 20 and also HIRSCHLER and AMON (l.c.), who have constated the presence of non-separable mixtures for a large number of hydrocarbon systems. If the index-level, on the other hand, shows variations which lie outside the tolerance of the refractometer, this may, for the fluids mentioned here, be referred to inhomogeneities in these fluids.

On the connection between wetting heat and adsorption affinity

GRIMM and co-workers, who as early as 1928 (19) showed that partial separation between a number of solvent components could be obtained with a sort of frontal analysis, presumed (20) that there was a close connection between wetting heat and affinity. Thus they found the wetting heat in cal/g (for fine-grained SiO_2) for C_6H_{12} : 6.25, $C_6H_5 \cdot CH_3$: 11.3, C_6H_6 : 11.3 and CH_3CH_2OH : 23.7. By means of the displacement technique it has also been proved that C_6H_{12} and C_6H_6 (or $C_6H_5 \cdot CH_3$) are very easily separable, and that ethanol displaces these quantitatively (1, 2). Benzene and toluene, on the other hand, are very difficultly separable (see Fig. 12). On the passage of the fluid through the adsorption column heat is developed, and this effect has been exploited by MAIR and co-workers (7) for the localization of the fluid front in iron or steel columns. No more detailed investigations of the temperature variation in the fluid-filled part of the adsorption column seem to have been carried out. To get some idea of this, a thermal element, alumel-chromel, was introduced in the lower part of a glass column, outer diameter 5 mm, inner diameter 2.5 mm, and packed in silica gel (DAVISON, through 200 mesh). Displacement adsorption was then carried out in the usual way. The reading off of the temperature as a function of mV was begun just before the fluid front reached the welding place, and thereafter every 5th second until the displacer (absolute ethanol) oozed out of the lower part of the column. The temperature-time diagrams thus obtained (Fig. 27) clearly confirm the close connection foreseen by GRIMM and co-workers between affinity and wetting heat. The curves also illustrate clearly the energetic principle for displacement adsorption. This method of recording—subject to further development—may also be conceived to be of use in the recording of inhomogeneities and as a complement to the index-measurement.

Some investigations on terpenes

In the sequence series, Table I, only a few of those fluids have been included which belong to the large group of "solvents", to which also the terpenes belong.

This group of fluids, however—which from industrial hygiene viewpoint is of special interest owing to the effect on human skin (turpentin-dermatitis)—

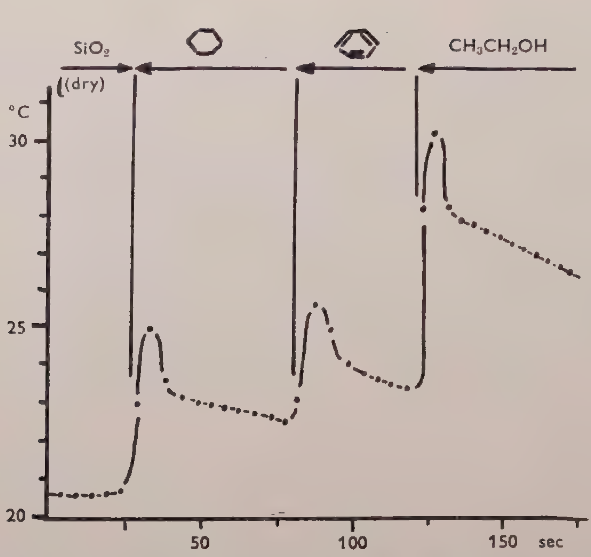


Fig. 27. Temperature variation in the silica gel on separation of a benzene-cyclohexane mixture. Displacer: absolute ethanol. The arrows connote visually observed fronts in the column.

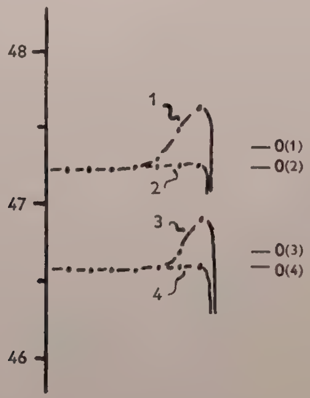


Fig. 28. Terpene analysis. 1. *l*-Limonene (KAHLBAUM), vacuumdistilled. 2. (1) adsorption purified. Level n_D^{20} : 1.4723. 3. α-Pinene (Bergvik & Ala), vacuumdistilled. 4. (3) adsorption purified. Level: n_D^{20} : 1.4658.

has been found wellsuited for examination with displacement adsorption. A preliminary report of some experimental results will be given here.

On testing the purity of some terpenes it proved that characteristic "peaks" where obtained at the end of the adsorptograms, Fig. 28. For these examinations the same apparatus dimension was used as for the sequence experiments. Absolute ethanol was used as displacer.

The nature of these "peaks" which are of interest because here a crowding of exematogenously active substances has been found to have taken place (21) is the object of further investigations. That it is presumably not a matter of products formed on contact between the terpenes and the adsorbent—for several acyclic olefines HIRSCHLER and AMON (l.c.) have observed that they form products of polymarysation in contact with silica gel—has been showed for α-pinene and l-limonene.

If the first fractions of these liquids with even index-level are separated from the "peaks" (purification by adsorption) and then analysed again one obtains no changes in the sample level recordable with refraction index.

SUMMARY

Supplementary technical details are given concerning a microtechnique for the investigation of liquids (solvents) with displacement adsorption on silica gel. Experimental data are presented which aim at illustrating the range of usefulness of the method. A number of solvent components comprising the lower homologues for hydrocarbons, acetates, ethane and ethene chlorinated hydrocarbons as well as methane chlorinated hydrocarbons, have been investigated with reference to their mutual affinities in the concentration 1:1. A sequence series has been drawn up and discussed from the practical point of view. Further, some preliminary experimental data about the connection between wetting heat and adsorption affinity and from investigations of terpenes have been communicated.

Each of the liquids has been tested with regard to the possibility that it may undergo changes in the adsorbed state. With the experimental conditions used no such changes recordable with refraction index have been observed.

The microtechnique in the form presented here—required amount of sample about one drop, time for analysis about 15 minutes—is well suited to give quick information concerning the homogeneity of a fluid, on condition that its components have different refraction indices and adsorption affinities.

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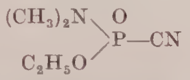
Biochemical studies with Tabun and allied compounds

By KLAS-BERTIL AUGUSTINSSON

With 9 figures in the text

Tabun or dimethylamido-ethoxy-phosphoryl cyanide



$$\begin{matrix}(CH_3)_2N\diagdown & O \\ & \rangle P—CN \\ C_2H_5O\diagup & \end{matrix}$$

is one of the most toxic organophosphorus compounds known; indeed, it is probably one of the most toxic compounds ever synthesised. Like most of the organophosporus poisons, Tabun is a highly active "anticholinesterase" drug; its pharmacological and toxic effects are most probably due to its inhibiting effect on the enzymatic acetylcholine hydrolysis (1).

The synthesis of Tabun can be traced back to SCHALL (2) and MICHAELIS (3), but the true chemical constitution of the compound was not recognized. The pioneer work of Dr. GERHARD SCHRADER (4) on toxic phosphorus compounds included the synthesis of Tabun and many of its analogues, which was patended in Germany July 22, 1937. Due to war restrictions, however, this synthesis was unknown until recently when the patent (767,511) was released for publication (Sept. 8, 1952); see note on p. 350. The formula of Tabun was published in Switzerland in 1948 (6).

In a recent publication by HOLMSTEDT (7) the chemical, biochemical, and pharmacological properties of Tabun have been extensively discussed; the synthesis described is based on the general method introduced by SCHRADER. HOLMSTEDT and LARSSON (8) made an infra-red spectroscopical examination of the compound and definitely established its formula. The hydrolysis of Tabun was studied by LARSSON who showed, by means of a qualitative infra-red spectrophotometric method (9) that in this process both the dimethylamino and the cyanide group are split off, but that splitting off of the ethoxy group is negligible. More recently on the basis of quantitative experiments made at various pH values LARSSON (10) discussed the mechanism of the hydrolysis.

HOLMSTEDT (7) attributes the pharmacological effects of Tabun to its cholinesterase inhibiting activity. A direct relationship was demonstrated between this activity *in vitro* and the pharmacological effects and toxic symptoms. Studies on the action of Tabun on spinal reflexes, reported by SKOGLUND and HOLMSTEDT (11, 12), showed that small doses (5–10 micrograms per kg body weight) produce definite and constant changes in central synaptic transmission. It is interesting that various reflex systems behaved differently, and that interneuron activity is especially influenced (35, 36). The same series of investigations also includes a study of muscle fasciculations (37).

Toxicological investigations with ^{32}P labelled Tabun have been briefly reported by GREPPIN (13) who found that the condition of intoxicated animals improved appreciably, and death was prevented after lethal doses in rabbits, by the administration of a mixture of an ester of *p*-aminobenzoic acid, atropine, and a benzylic ester. The protection of cholinesterases *in vitro* by procaine was demonstrated by AUGUSTINSSON (14) who also found that Tabun reacts with certain dihydroxyphenyl derivatives (15; see below).

The *in vivo* protective effect of some phenothiazine derivatives has been reported recently by DAHLBOM *et al.* (16).

The effect of Tabun on the central vestibular mechanism has been investigated by DIAMANT (17). By a centripetal intracarotal injection technique (guinea pigs) Tabun, reaching the vestibular nuclei, produced a typical circling syndrome which could be counteracted by atropine both prophylactically and therapeutically. In these experiments the esterase activity was determined electrometrically (18).

A full account of the enzyme studies made in this laboratory between 1949 and 1952 will be presented in the present paper.

Methods and material

Measurement of enzyme activity. The WARBURG manometric technique was used to measure rates of acetylcholine hydrolysis (19). All measurements were made at 25°C. The esterase activity is expressed in b_{30} values, *i.e.*, the amount of CO_2 in µl evolved from a bicarbonate solution (see below) during 30 minutes minus the corresponding value for non-enzymatic hydrolysis.

The esterase activity at various pH was determined by a colorimetric method, described by HESTRIN (20). This method is based on the interaction of unhydrolysed acetylcholine with alkaline hydroxylamine to form acethydroxamic acid which forms a coloured complex with ferric ions.

Buffer solution. Substrate, enzymes, and inhibitors were dissolved in Ringer's bicarbontate solution containing 0.12 M NaCl, 0.034 M $NaHCO_3$, and 0.0012 M $MgCl_2 \cdot 6H_2O$.

Enzyme preparations:

Plasma and erythrocytes. Human blood was taken in heparinized test tubes. The red cells were separated by centrifugation and washed four times with 0.9 per cent sodium chloride solution. The cells were haemolysed to blood volume with distilled water. When used in inhibition experiments, this haemolysate was further diluted with two parts of bicarbonate buffer solution. The plasma was diluted with 0.5 parts of the same solution.

Thrombocytes were isolated from the blood of cats, washed twice in saline solution, and then suspended in a suitable volume of distilled water and finally diluted with bicarbonate solution.

Nucleus caudatus was dissected out from dog's brain, homogenized, and suspended in bicarbonate solution. The whole suspension was used as enzyme preparation.

Electric tissue was prepared from the electric ray (*Torpedo marmorata*). The electric organs were cut into small pieces and treated with toluene for five weeks to remove most of the appreciable amount of mucin present; the exudate containing mucin was discarded periodically. At the end of the five weeks, the material was freeze dried. This dry material is very stable when kept in a dry vessel, even at room temperature; the material kept in this way for two years did not lose any activity. The material when used was suspended in bicarbonate solution (centrifugation not necessary), 10 mg in 10 ml. This preparation contains acetylcholinesterase alone as choline-ester splitting enzyme and is free of aliesterases.

Cobra venom. A dried and crystallised preparation from *Naja naja* was employed. It was dissolved in bicarbonate solution, 5 mg in 10 ml.

Substrate. Acetylcholine chloride was used as substrate in all experiments, the concentration being $1.10 \times 10^{-2}\ M$ for plasma butyrylcholinesterase and $3.30 \times 10^{-3}\ M$ for the acetylcholinesterases (erythrocytes, nucleus caudatus, cobra venom, electric tissue), unless otherwise stated; total volume of the reaction mixture, 2.00 ml.

Tabun and other organophosphorus compounds have been synthesised at the Research Institute of National Defense, Department 1, in Sweden, under the leadership of Dr. Bo Holmstedt (7). TEPP (tretraethyl pyrophosphate and DFP (di*iso*propylfluor phosphate) were given by the Chemical Department, Army Chemical Center, Maryland, USA. They have all been of high purity. The physical constants of Tabun were: b.p. 53–55°C/1 mm; $d^{20} = 1.077$.

Other compounds used. A great many compounds were used as protectors against enzyme inactivation by Tabun. With the exception of physostigmine salicylate and prostigmine bromide, they are all listed in Table 4 and 5. The local anaesthetics and other compounds employed more extensively in such experiments were the following (cf. Table 5): benzocaine (ethylamino benzoate), pantocaine (tetracaine hydrochloride, the 2-dimethyl-aminoethyl ester of *p*-butyl-aminobenzoic acid), parpanit (caramiphen hydrochloride, 1-phenylcyclopentane carboxylic acid 2-diethyl-aminoethyl ester hydrochloride), percaine (dibucaine hydrochloride, 2-butoxy-N-(2-diethyl-aminoethyl) cinchoninamide hydrochloride), procaine (novocaine hydrochloride), stovaine (amylocaine, 2-(*p*-aminobenzoxy)-1-*n*-amylaminoethane hydrochloride), tutocaine hydrochloride (3-dimethylamino-1,2-dimethyl-propyl *p*-aminobenzoate hydrochloride, xylocaine hydrochloride (α-diethylamino-2,6-aceto-xylidide); diparcol (2987 R. P., 10-(2-diethyl-aminoethyl) phenothiazine), multergan (3554 R. P., N-/β-(10-phenothiazinyl) propyl/trimethylammonium chloride).

In vivo experiments. A series of experiments were carried out *in vivo* with Tabun and some other allied compounds. Dogs were injected subcutaneously with 0.43 μmole per kg body weight of the compounds to be tested. Blood samples were taken before and 10–15 minutes after the injection and then periodically after various lengths of time. Plasma and erythrocytes were separated and the esterase activity determined against acethylcholine in the manner described above.

Results

Comparison between the cholinesterase inhibiting effect of organophosphorus compounds in vitro. In comparing the "anticholinesterase" activity of various compounds, human erythrocytes were used as the enzyme source. The acetylcholine concentration in all these early experiments was $1.10 \times 10^{-2}\ M$. The enzyme solution was incubated for 60 minutes with various concentrations of the inhibitor (inactivator). The esterase activity of these mixtures was tested by taking 0.4 ml (in the side bulb of the Warburg vessel) and 1.6 ml of the substrate solution (in the main compartment). It will be noted that in this procedure the inhibitor-esterase mixture is diluted five times when the esterase activity is determined. There is no true equilibrium between inhibitor, enzyme and substrate, because the reaction between an organophosphorus compound and cholinesterase is more or less irreversible. For this reason it is more correct to state that concentration of the inhibitor (being actually an inactivator) which is present during incubation and not that one present during esterase determination, *i.e.*, five times lower in the experiments described (Fig. 1).

The inhibiting effect is expressed in the molar concentration of the inhibitor, I_{50}, which gives 50 per cent inhibition of a standard enzyme preparation; pI_{50}, which gives 50 per cent inhibition of a standard enzyme preparation; pI_{50} is the negative logarithm of I_{50}. This value is obtained graphically by extrapolation. The concentra-

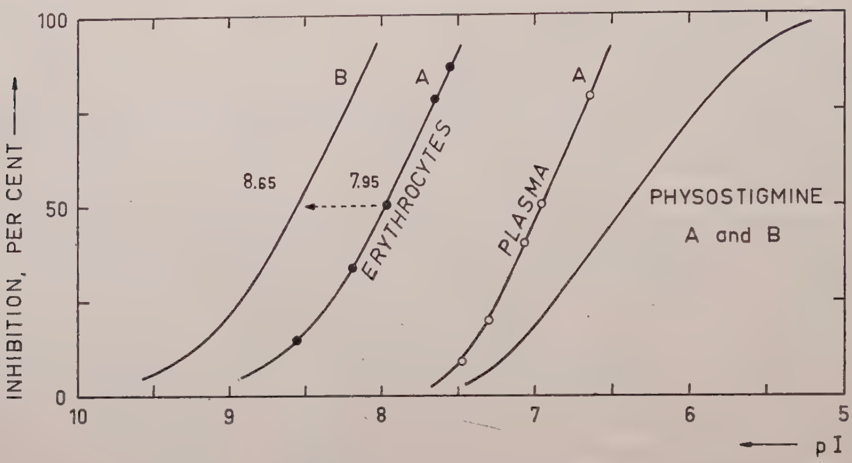


Fig. 1. Per cent inhibition (inactivation) of erythrocyte (human) acetylcholinesterase by Tabun compared with the Tabun inhibition of the acetylcholine hydrolysis by human plasma. The inhibition of purified electric eel acetylcholinesterase by physostigmine (a typical reversible and competetive inhibitor; data from the work of AUGUSTINSSON and NACHMANSOHN (21)) is inserted for comparison. Incubation period, 60 minutes. A: during incubation; B: during esterase determination.

tion of the inhibitor is plotted against the inhibition, expressed as v/v', where v is the initial reaction velocity in the absence of the inhibitor, v' in its presence. Both v and v' are expressed in b_{30} values. This method has been described in detail in a previous publication (19).

The principle of the method is illustrated in Fig. 2, which demonstrates the inhibiting effect of bis(dimethylamido)-phosphoryl fluoride. The effect is comparatively low unless the toxicity is known to be very high (7). Most probably, this compound is converted to a powerful cholinesterase inactivator in the organism and by tissue extracts. Preliminary incubation experiments with liver extract and plant material strongly support this view. Juice of plants given water alone and water containing bis(dimethylamido)-phosphoryl fluoride (this solution was without inhibiting effect on cobra venom acetylcholinesterase) was prepared. It was shown that the juice from the plant treated with the toxic solution had esterase inhibiting effect compared with control. The inhibiting effect of the compound is progressive and a bicarbonate solution of it loses its effect due to hydrolysis.

Table 1 summarises the results obtained with various organophosphorus compounds and human erythrocyte acetylcholinesterase. The most active compound studied is dimethylamido-*iso*propoxy-phosphoryl cyanide, which is still more active than the ethoxy analogue (Tabun). In the group of amido substituted phosphoryl compounds the cyanides are more potent inactivators than the chlorides (or fluorides). For comparison between cholinesterase inactivating effect and toxicity (to albino mice) the LD_{50} values, reported by HOLMSTEDT (7), are included in Table 1.

The inhibition of various types of cholinesterases by Tabun. The inhibition of cholinesterases from various sources by Tabun is recorded in Table 2. The results were obtained in the manner described in the foregoing section. Tabun inactivates the

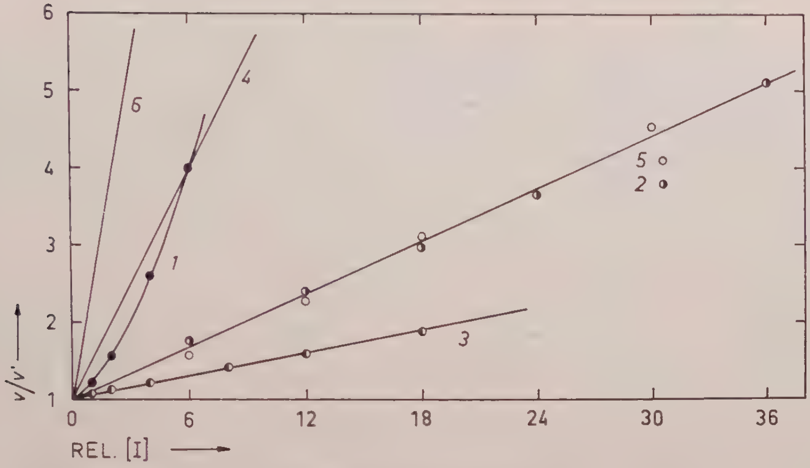


Fig. 2. Enzyme inhibition by bis(dimethylamido)-phosphoryl fluoride (DMF) as function of inhibitor concentration. v = velocity in the absence, v' = velocity in the presence of DMF (expressed in b_{30} values). Relative inhibitor concentration (Rel. [I], 1 = 3.02×10^{-4} *M* during enzyme incubation (0.604×10^{-4} *M* during esterase determination).

Curve No.	Enzyme	Age of DMF solution in buffer	Incubation period	I_{50} [1]	pI_{50} [1]
1	Plasma (human)	45 min.	60 min.	0.87×10^{-3}	3.06
2	Erythrocytes (human)	25 min.	60 min.	2.65×10^{-3}	2.58
3	»	3 hours	60 min.	6.00×10^{-3}	2.22
4	»	25 min.	24 hours	0.60×10^{-3}	3.22
5	Cobra venom	25 min.	60 min.	2.65×10^{-3}	2.58
6	»	25 min.	24 hours	0.18×10^{-3}	3.75

[1] The I_{50} (pI_{50}) values refer to molar concentration of DMF in the incubation mixture.

acetylcholinesterases from various sources to the same extent, *i.e.*, the cholinesterase type found in erythrocytes, thrombocytes, nucleus caudatus, electric tissue, and cobra venom. The pI_{50} value is 7.9–8.2. The progressive effect, as tested during various incubation periods, is somewhat different for the various preparations.

It will be seen from Table 2 that the inhibition of plasma (butyryl-)cholinesterase is less than that of the acetylcholinesterases. This difference observed between the two types of esterase is not due to a true selective difference in inhibition, but is the result of a "second" reaction between Tabun and other constituents of the plasma. The experimental basis for this conclusion will be reported elsewhere in due course. The values obtained for the inactivation of plasma cholinesterase, shown in Fig. 1 and Tables 2 and 3, therefore do not give the true values for this enzyme. When the inhibiting power of Tabun is determined for comparison by both the gasometric (Warburg) and the electrometric technique (Michel; 18), the values obtained differ considerably regarding the plasma enzyme (Table 3). This difference is due to the "second" reaction mentioned.

Table 1. The inhibiting action of various organophosphorus compounds on human erythrocyte acetylcholinesterase. Substrate: acetylcholine chloride, 1.10×10^{-2} *M*. I_{50} = molar inhibitor concentration giving 50 per cent inhibition; pI_{50} = negative logarithm of this concentration; A: during incubation; B: during esterase determination. The enzyme was incubated 60 minutes with the inhibitor before mixed with the substrate. — The LD_{50} values µmol/kg body weight, are those reported by HOLMSTEDT (7); they are obtained by intraperitoneal injection in albino mice.

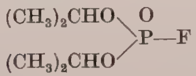
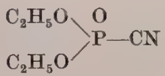
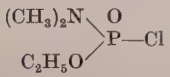
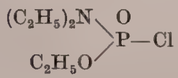
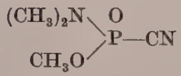
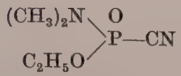
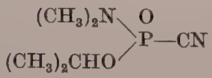
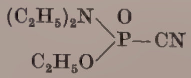
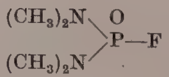
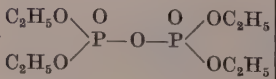
Formula	Name or code	Mol. wt.	Density g/ml 20°C	I_{50}	pI_{50}		LD_{50}
				A	A	B	
 $((CH_3)_2CHO)_2P(O){-}F$	DFP	184.15	1.070	3.89×10^{-7}	6.41	7.11	60.61
 $(C_2H_5O)_2P(O){-}CN$	Diethoxy-phosphoryl cyanide	163.12	1.084	4.20×10^{-7}	6.38	7.08	8.25
 $(CH_3)_2N(C_2H_5O)P(O){-}Cl$	Dimethyl-amido-ethoxy-phosphoryl chloride	171.57	1.188	1.24×10^{-5}	4.91	5.61	—
 $(C_2H_5)_2N(C_2H_5O)P(O){-}Cl$	Diethylamido-ethoxy-phosphoryl chloride	199.63	1.120	2.52×10^{-5}	4.60	5.30	—
 $(CH_3)_2N(CH_3O)P(O){-}CN$	Dimethylamido-methoxy-phosphoryl cyanide	148.11	1.12	8.15×10^{-8}	7.09	7.79	12.92
 $(CH_3)_2N(C_2H_5O)P(O){-}CN$	Tabun (cf. Fig. 1)	162.13	1.077	1.08×10^{-8}	7.95	8.65	3.73
 $(CH_3)_2N((CH_3)_2CHO)P(O){-}CN$	Dimethylamido-*iso*-propoxy-phosphoryl cyanide	176.16	1.054	6.35×10^{-9}	8.20	8.90	2.62
 $(C_2H_5)_2N(C_2H_5O)P(O){-}CN$	Diethylamido-ethoxy-phosphoryl cyanide	190.19	1.029	1.42×10^{-7}	6.85	7.55	21.24
 $((CH_3)_2N)_2P(O){-}F$	Bis(dimethylamido)-phosphoryl fluoride (cf. Fig. 2)	154.10	1.115	2.65×10^{-3}	2.58	3.28	7.53
 $(C_2H_5O)_2P(O){-}O{-}P(O)(OC_2H_5)_2$	TEPP	290.20	1.185	1.60×10^{-7}	6.79	7.49	2.54

Table 2. Inhibition of cholinesterases from various sources by Tabun *in vitro*. The I_{50} values refer to molar concentration of Tabun in the incubation mixture kept at 4.5°C. During esterase determination the Tabun concentration is 5 times lower. The I_{50} values were determined after 60 minutes, 3 days and 7 days of incubation.

Enzyme source	Incubation period					
	60 minutes		3 days		7 days	
	I_{50}	pI_{50}	I_{50}	pI_{50}	I_{50}	pI_{50}
Plasma (human)	1.07×10^{-7}	6.97	1.02×10^{-7}	6.99	0.96×10^{-7}	7.02
Erythrocytes (human)	1.08×10^{-8}	7.95	1.03×10^{-8}	7.98	1.05×10^{-8}	7.98
Thrombocytes (cat)	10.3×10^{-9}	7.98	7.6×10^{-9}	8.12	5.6×10^{-9}	8.25
Nucleus caudatus (dog)	15.4×10^{-9}	7.81	8.8×10^{-9}	8.06	8.85×10^{-9}	8.05
Electric tissue (*Torpedo marmorata*)	10.5×10^{-9}	7.98	7.20×10^{-9}	8.14	5.4×10^{-9}	8.27
Cobra venom (*Naja naja*)	6.1×10^{-9}	8.21	6.1×10^{-9}	8.21	5.7×10^{-9}	8.24

It will also be noted that the inhibiting action expressed in I_{50} values is not the same for various blood samples. This is illustrated in Table 3 for the three different human blood samples tested.

Table 3. Inhibition of plasma and erythrocyte cholinesterases by Tabun expressed in pI_{50} values, calculated for the incubation and reaction mixtures. Three blood samples (human) tested, one of which was used simultaneously for electrometric esterase determinations in comparison with the gasometric ones. Incubation period, 60 minutes.

Enzyme source	1	2	3			
			Incubation mixture		Reaction mixture	
			Gasom.	Electrom.[1]	Gasom.	Electrom.[1]
Plasma	6.97	7.35	6.83	8.08	7.53	8.12
Erythrocytes	7.95	7,70	7.85	8.58	8.55	8.63

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[1] Determinations made by Dr. L.-E. Tammelin (cf. 18).

Inhibition of acetylcholinesterase by Tabun as function of pH. In testing the inhibiting action of Tabun on cobra venom acetylcholinesterase the colorimetric method of Hestrin (20) was used. The enzyme activity at various pH was determined in the presence (v') and in the absence (v) of Tabun, and the v/v' value calculated. The results are recorded in Fig. 3 where this value is plotted against pH. For comparison the activity-pH curve is inserted; cf. the pH dependence of this enzyme reported by Grauers (22).

The activity of Tabun as an inhibitor of acetylcholinesterase shows a dependence on pH which is similar to the enzyme activity when tested against acetylcholine. The result is in agreement with the view that the phosphate esters react with the esterase in a way similar to the reaction between acetylcholine and the enzyme, *i.e.*,

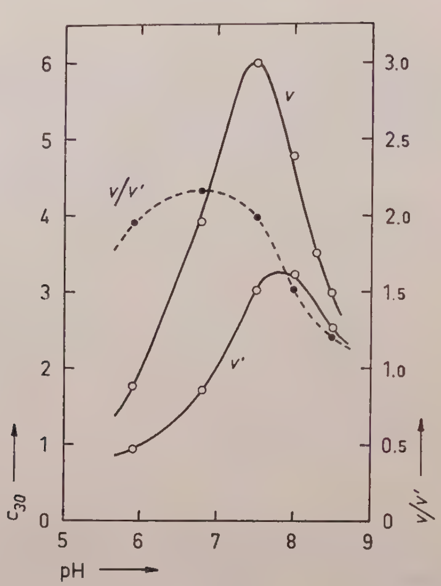

Fig. 3. Inhibition of cobra venom acetylcholinesterase by Tabun as function of pH and activity-pH curve of the enzyme. Activities expressed in c_{30} values, *i.e.* μmol acetylcholine hydrolysed in 30 minutes. v and v' (see Fig. 2), expressed in c_{30} values.

the phosphorylation by phosphate esters is analogous to the acetylation by acetylcholine. Working with a purified preparation of electric tissue, NACHMANSOHN and WILSON (23) reported similar results with TEPP and concluded that "those changes in the protein which favor hydrolytic activity also favor the irreversible reaction with TEPP".

The irreversibility of Tabun inhibition in vitro. The inhibiting action of organophosphorus compounds on cholinesterases is in most cases an irreversible process, but recent experiments have demonstrated that certain esterases inhibited by some of these compounds are reactivated, the velocity of this process generally being comparatively slow and dependent on the experimental conditions. Thus, the TEPP-inhibited esterases of various sources were reactivated (24, 25); the lower homologues of "dialkylphosphostigmines" are also reversible inhibitors (26).

Esterases inhibited by Tabun has not been found to be reactivated. The enzyme preparation was incubated with Tabun at pH 7.4. The concentration of the inhibitor was chosen to give about 60 per cent inhibition. The enzyme solutions together with controls were kept in the refrigerator (4.5° C) and at 25° C respectively. The esterase activity was measured after one hour and then periodically for 36 days. The results obtained with cobra venom are illustrated in Fig. 4. No reactivation of the enzyme activity could be observed. The reactivation of the same enzyme after inhibition by TEPP is included for comparison.

Protection against Tabun inactivation. It has been shown previously (see review by the present author, 27) that certain compounds protect cholinesterases against irreversible inhibition (*i.e.*, inactivation) by organophosphorus compounds *in vitro*

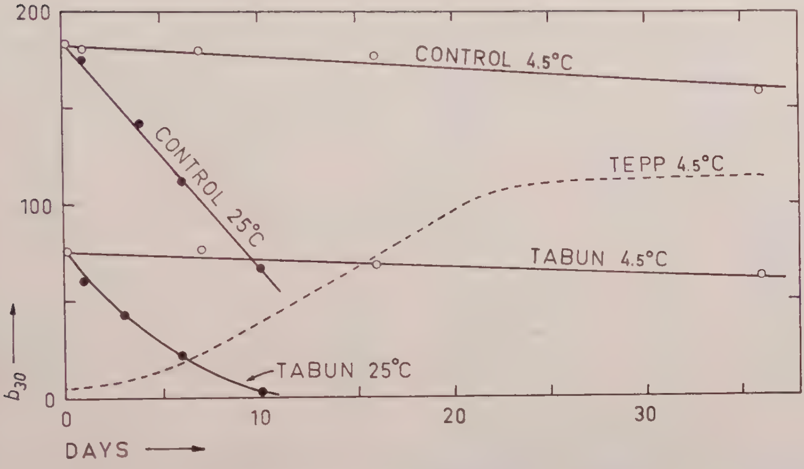

Fig. 4. Cobra venom acetylcholinesterase treated with Tabun and kept at various temperatures. The reactivation curve of the same enzyme inhibited by TEPP is inserted for comparison (25).

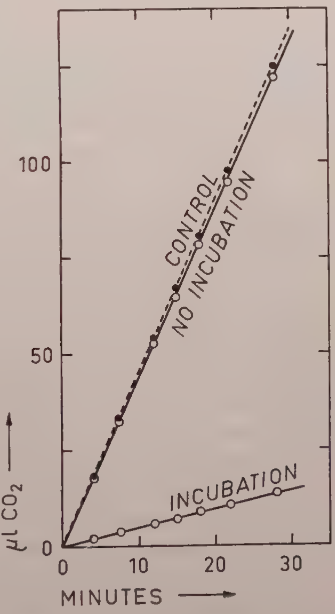

Fig. 5. The enzymatic hydrolysis of acetylcholine and the inhibition of this reaction by Tabun. Enzyme: erythrocyte haemolysate (human). Control: no Tabun. Incubation: the enzyme incubated 60 minutes with Tabun before acetylcholine was added. No incubation: enzyme in contact with Tabun and acetylcholine simultaneously. Tabun and substrate concentrations in the reaction mixtures, 8.5×10^{-8} *M* and 1.10×10^{-2} *M* respectively. Corrections made for spontaneous substrate hydrolysis.

and *in vivo*. Any compound reacting with the active centre of the enzyme molecule which is irreversibly destroyed by an organophosphorus compound is a protector. Thus, acetylcholine, physostigmine, prostigmine, etc. might be useful protectors. The results with Tabun are reported below.

Acetylcholine. When the enzyme is first treated with acetylcholine, a concentration of Tabun several times greater than usual is necessary for inhibition. This enzyme protection by the substrate is demonstrated in Fig. 5 with erythrocyte

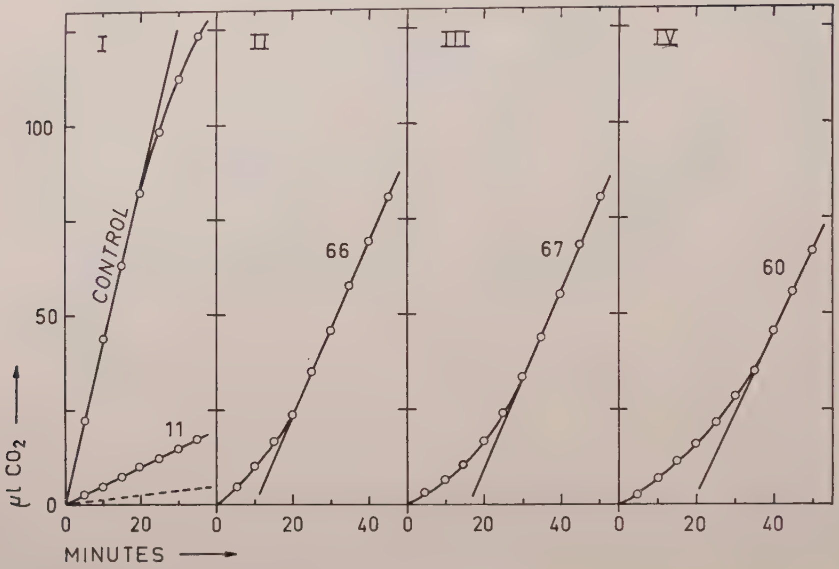

Fig. 6. Protection of cobra venom acetylcholinesterase against inactivation by Tabun. Protector: prostigmine bromide. Numbers refer to esterase activity (b_{30}). Broken line: nonenzymatic hydrolysis. I: control experiment and enzyme incubated with Tabun. II: enzyme incubated with prostigmine. III: enzyme mixed with prostigmine *before* incubation with Tabun. IV: Tabun and prostigmine simultaneously mixed with the enzyme. Concentrations in the reaction mixture: acetylcholine, 3.3×10^{-3} *M*; Tabun, 6.0×10^{-9} *M*; prostigmine, 4.6×10^{-9} *M*.

acetylcholinesterase. That concentration of Tabun which gives 90 per cent inhibition in the incubation experiments has practically no effect when it is mixed with the enzyme at the same time as the substrate (acetylcholine).

Physostigmine and prostigimine protect cholinesterases against inactivation by Tabun. The protection of cobra venom acetylcholinesterase by prostigmine is illustrated in Fig. 6. The concentration of Tabun was chosen to give about 90 per cent inhibition, that of prostigmine to give about 50 per cent inhibition. It will be seen from the figure that Tabun has no effect on the prostigmine treated enzyme (III). Even when prostigmine and Tabun are simultaneously mixed with the enzyme (IV), the greater affinity of prostigmine for the enzyme affords protection against enzyme inactivation by Tabun. Similar results have been obtained with physostigmine and other cholinesterase preparations.

Other compounds. A great many compounds have been tested as protectors. In the early experiments of this kind TEPP was used as inhibitor, in the later ones Tabun was employed. Cobra venom was generally used as enzyme preparation, but other enzyme preparations have also been tried.

The results of the preliminary experiments with TEPP are recorded in Table 4, the first part (A) of which lists those compounds which have not been found to be active protectors; in the second part (B) compounds exerting protective effects are listed. All compounds with protective effect combine with the same active centre of the enzyme molecule as do the irreversible inhibitors (*e.g.*, TEPP or Tabun) and they have all a demonstrable inhibiting effect on the enzyme.

Table 4. Compounds tested in preliminary experiments as protectors of cobra venom acetylcholinesterase against inactivation by TEPP. Optic isomers are used as DL-forms, unless otherwise stated. Values represent per cent enzyme inhibition by 5.0×10^{-3} M of the compounds tested. A: non-protecting compounds; B: protecting compounds.

Compound	Per cent inhibition	Compound	Per cent inhibition
		A.	
Sarcosine	21	Neurine bromide	36
α-Alanine	4	Urethane	3
β-Alanine	0	Biuret	0
Serine	0	Creatinine	3
Valine, DL	6	Spermine phosphate	13
» L	6	Agmatine sulphate	0
» D	5	Diacetyl	5
Leucine, DL	8	Gycerophosphate, Na-	0
» L	16	Acetylsalicylic acid	17
» D	0	Nicotinic acid	0
Aspartic acid	4	Trigonelline	0
Glutamic acid	6	Methylene blue	100
Ornithine 2 HCl	7	Barbituric acid	75
Arginine	0	Phenobarbital	33
Tryptophane, L	5	Caffeine	62
» D	0	Sulphanilamide	0
Proline, DL	0	Diacetylmorphine	13
» L	0	Quinacrine (Atabrine)	86
Hydroxyproline, L	0	Quinine HCl	27
Cysteine, DL	85	Pilocarpine HCl	73
» L	80	Tubocurarine chloride	80
Cystine	0	Gum arabic, 2.0 mg	0
Glutathione, reduced	74	Snake venom, Viperidae, 2.0 mg	0
		B.	
p-Aminobenzoic acid	42	Carbamylcholine chloride	79
Histamine 2 HCl	35	N-Acetyl-*p*-aminobenzoylcholine chloride	52
Guanidine HCl	4	Succinylcholine diiodide	74
Procaine HCl	66	Clupeine, 0.2 mg	58
α,α′-Dipyridyl	14	Bee venom, 2.0 mg	8
Atropine sulphate	15		

Among the non-protecting compounds (Table 4 A) it will be noted that cystein and reduced glutathione inhibit the cobra venom esterase. No protection occurs, however, which is due to the reaction of cystein with a group in the enzyme molecule other than that combining with TEPP. Similar observations were made with neurine, methylene blue, nicotinic acid, barbiturate, caffeine, quinacrine, pilocarpine, and tubocurarine.

It was recently announced (28) that acetylcholinesterase (electric eel) inactivated by DFP gives serine phosphoric acid on hydrolysis. Phosphorylation of serine *in vitro* by TEPP, however, has not been accomplished in the present investigation (Table 4 A).

Many choline esters are active protectors against TEPP. In addition to those

Table 5. Protection of cholinesterases against Tabun by different compounds. Hydrochlorides of the bases used, unless otherwise stated. Substrate: acetylcholine chloride. Enzyme preparations treated in the following various ways: I: incubation with Tabun for 60 minutes; II: incubation with protector; III: enzyme mixed with the protector before incubation with Tabun for 60 minutes; IV: Tabun and protector mixed together and the enzyme incubated for 60 minutes with this mixture. Per cent protection: $\frac{\text{III}-\text{I}}{\text{II}-\text{I}} \times 100$.

Protector	Enzyme	Concn. of protector M	Control	I	II		III		IV
				b_{30}	b_{30}	% inhib.	b_{30}	% protect.	b_{30}
Procaine	Plasma	5.0×10^{-5}	157.5	6	157.5	0	110.5	69	110
»	Erythrocytes	5.0×10^{-5}	155	8	155	0	28.5	15	30.5
»	Cobra venom	5.0×10^{-4}	177	13.5	143	19	73.5	46	42
Benzocaine . . .	Cobra venom	5.0×10^{-4}	185	15	169	8.5	16	nil	—
Tutocaine	Cobra venom	5.0×10^{-5}	164	9.5	48	71	31	57	33
»	» »	5.0×10^{-6}	148	12.5	122.5	17	29	15	31
Pantocaine . . .	Plasma	6.2×10^{-6}	148.5	4	118	21	8	3	—
» . . .	Erythrocytes	1.2×10^{-5}	110	0	93	16	15	16	—
» . . .	»	5.0×10^{-6}	116	3	110	5	16	12	14
» . . .	»	2.5×10^{-6}	110	0	107	3	3	nil	—
» . . .	Cobra venom	5.0×10^{-5}	178	21	151	15	71	33	55
Stovaine	Cobra venom	5.0×10^{-4}	126	5.5	77	39	20.5	21	14
Percaine	Cobra venom	5.0×10^{-3}	171	11	9	95	10	nil	—
»	» »	5.0×10^{-5}	174	8	108	38	10.5	nil	—
Xylocaine	Cobra venom	5.0×10^{-3}	174	30	91.5	47	45.5	25	47
»	» »	5.0×10^{-4}	121	24	104.5	14	21	nil	22
»	» »	5.0×10^{-5}	110	2	95	14	3	nil	—
Parpanit	Plasma	2.0×10^{-6}	121.5	39	87	28	40	nil	44
»	»	5.0×10^{-7}	143.5	3.5	131	9	8	nil	8
»	Erythrocytes	7.5×10^{-4}	126.5	2	106	16	23	20	26
»	»	5.0×10^{-4}	119	2.5	105	12	28.5	25	29
»	»	2.5×10^{-4}	104.5	4	101.5	3	15	11	15.5
»	Cobra venom	5.0×10^{-3}	155	23	5	97	5.5	nil	5
»	» »	5.0×10^{-4}	176	9.5	146	17	50	30	49
Diparcol	Cobra venom	5.0×10^{-4}	178	39	102	42.5	41	nil	42
Multergan	Cobra venom	5.0×10^{-5}	172	36	122	29	34	nil	36
Atropine sulphate	Cobra venom	5.0×10^{-4}	158	12.5	153	3	23	8	22
» »	» »	5.0×10^{-5}	136	5	137	0	8	2	—
Pyrogallol	Cobra venom	5.0×10^{-4}	101	7	93.5	7	21	16	*51.5*

listed in Table 4 B butyrylcholine and propionylcholine are especially effective protectors (an observation also made recently for butyrylcholine by COHEN *et al.*, 29). Benzoylcholine and salicylcholine, on the other hand, are practically inactive. Choline itself is an active protector for some organophosphorus compounds, but not for all such compounds tested. The protective effect of choline varies also from enzyme to enzyme.

TEPP seems to react with α,α′-dipyridyl and a small protective effect was ob-

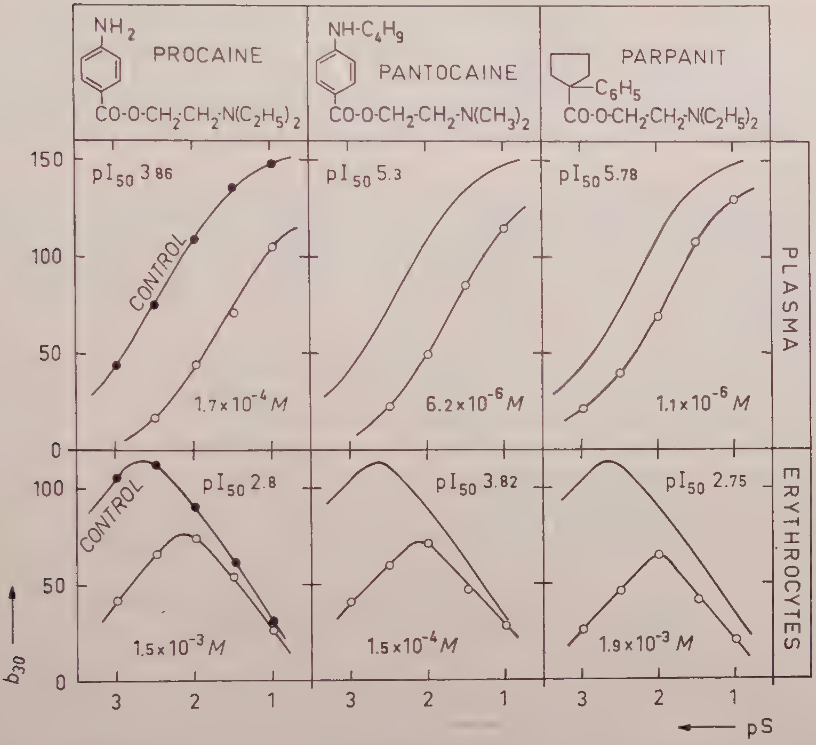

Fig. 7. The inhibition of enzymatic acetylcholine hydrolysis by three compounds found to have protective action against enzyme inactivation by Tabun. For each enzyme and compound the inhibiting effect is shown for a certain inhibitor concentration as function of substrate concentration (pS = —log [S]).

served. This confirms a recent finding (30) that the hydrolysis of DFP is enhanced by α,α′-dipyridyl especially when small amounts of certain metals are present.

In these early experiments with TEPP it was observed (Table 4 B) that procaine protects the cobra venom esterase against activation. A special study using procaine as a protector of cholinesterases against Tabun inactivation *in vitro* was carried out and the results have been reported recently (14). This effect is more pronounced for the plasma cholinesterase than for the acetylcholinesterases (see also Table 5). Details of the results obtained with procaine will be found in the paper cited; the properties of procaine as a choline esterase inhibitor are demonstrated in Fig. 7 of the present paper.

As a consequence of the above observation other local anaesthetics were tested as protectors. The results are recorded in Table 5. These compounds are inhibitors of cholinesterases of which the plasma cholinesterase is especially sensitive. Those compounds containing an ester linkage are enzymatically hydrolysed. The affinity of tutocaine and pantocaine for this esterase is comparatively high. Pantocaine has a marked protective effect against Tabun inactivation, which effect in the case of acetylcholinesterases is more pronounced than that shown by procaine.

The recently discovered xylocaine has a low inhibiting effect and its protective effect is negligible.

It is known from many recent studies (31) that parpanit is split by blood plasma It has been demonstrated conclusively in this laboratory that plasma cholinesteras is the enzyme responsible for this reaction, as it is for the enzymatic procaine hydrolysis. The protective effect against Tabun inactivation is very low for the plasm esterase; such an effect, however, is unequivocal for the acetylcholinesterases teste (Table 5).

Hence, the following four compounds have been found to be active esteras protectors: procaine, tutocaine, pantocaine, and parpanit. They are all both inhibitor and substrates of cholinesterases. The inhibiting properties of procaine, pantocaine and parpanit are illustrated in Fig. 7. They all behave as true competitive an reversible inhibitors for both the plasma and erythrocyte esterases when acetylcholin is used as substrate.

In addition to the compounds mentioned, two antihistaminics, diparcol an multergan, were tested as protectors, but neither exhibtis such effect (Table 5) Atropine is a weak protector against inactivation by Tabun.

In the series of experiments described it was observed that the "protector" i some cases reacts spontaneously with the inactivator (TEPP or Tabun). Pyrogallc was especially noticeable in this respect (Table 5) and this observation prompted th experiments, the results of which will be summarized in the next section.

Reaction of Tabun with adrenaline and other dihydroxyphenyl derivatives. A shor communication concerning the reaction of organophosphorus compounds with di and tri-hydroxyphenyl derivatives was recently published (15). This study include pyrogallol, pyrocatechol, hydroquinone, resorcinol, floroglucine, adrenaline, noradrenaline, and histamine. All these compounds react more or less spontaneously with DF in a bicarbonate solution with the evolution of CO_2. A similar observation was announced at the same time by JANDORF *et al.* (32).

Tabun also reacts with the same compounds in equimolecular proportions and i was demonstrated that the reaction products are phosphorylated dihydroxypheny derivatives. It has been found that the reaction products are much less active a cholinesterase inhibitors than Tabun (or DFP). The results obtained with the reac

Table 6. Inhibiting effect of the reaction products of adrenaline with Tabun an DFP on acetylcholinesterase (cobra venom). Concentrations of Tabun and DF during esterase determination, 2.5×10^{-3} *M*. Enzyme activity expressed in b_{30} values

Compound 5 μmol	Incubation period with 100 μmol adrenaline Hours	Controls; unreacted compound absent/present		Incubation mixture	
		absent	present	b_{30}	% inhibition
Tabun	1.5	159	0	9	96
	18	160	0	27	83
	21	154	0	35	77
	42	163	9	61	62
	69	169.5	14.5	71	58
DFP	4.5	167	0	3.5	98
	24	157.5	0	156	1

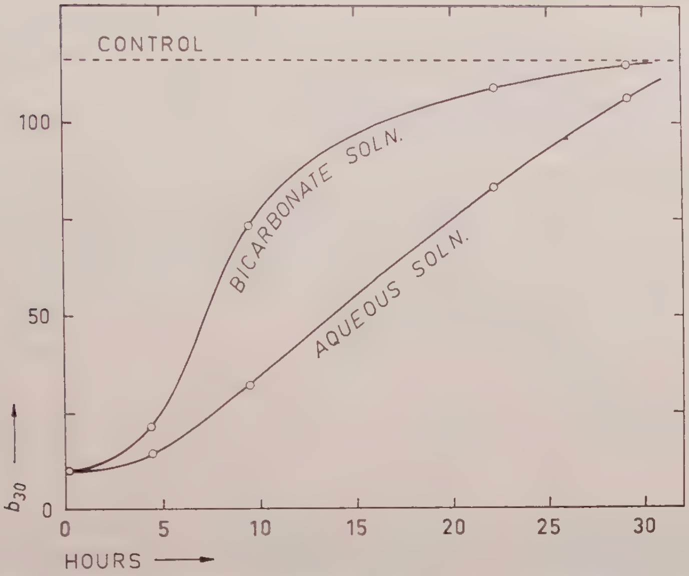

Fig. 8. Stability of Tabun solutions (0.001 per cent) at 25°C expressed in the inhibiting effect of the solutions on erythrocyte acetylcholinesterase.

tion product of Tabun and adrenaline are recorded in Table 6; one set of experiment carried out with DFP is included.

The reaction between the phenol derivatives and the organophosphorus compounds s a comparatively slow reaction. It may be, however, that the reaction is accelerated n the living body and that certain catalysts may be found (*e.g.*, heavy metals) which enhance this reaction even *in vitro.*

Stability of Tabun solutions. The behavior of Tabun in aqueous solutions of various pH has been studied by Larsson (9, 10). An early experiment carried out by the present author in order to find out the esterase inhibiting activity of Tabun solutions kept for various time periods is illustrated in Fig. 8. Tabun is hydrolysed in distilled water and more rapidly in bicarbonate solution of pH 7.8. It is known from the work by Larsson that at these pH values the P–CN linkage is susceptible to hydrolytic clevage.

It is practicable to have a stock solution of Tabun for experimental work. A propyene glycol solution is useful and such a 0.1 per cent solution is stable for a very long time if the glycol is absolutely free of water.

Distribution of Tabun between water and lipids. The solubility of Tabun in water and lipids is of fundamental importance of the understanding of the distribution of the compounds in living material. Most toxic organophosphorus compounds are eadily soluble in oils, more readily than in water. The distribution of Tabun between queous and oil phases was studied *in vitro* and one series of experiments is recorded n Table 7. An aqueous solution of Tabun (or TEPP), the enzyme inhibiting effect f which was known, was shaken with the same volume of peanut oil. After shaking

Table 7. Distribution of Tabun and TEPP between water and oil (peanut oil). Original aqueous solution of the compounds shaken with the same volume of oil. After separation of the two phases, their cholinesterase inhibiting activity was measured against cobra venom and expressed in per cent inhibition of control. Various concentrations of Tabun and TEPP used in the two sets of experiments.

Compound	Control	Shaking period Minutes	Enzyme incubated with					
			original soln.		aqueous phase		oil phase	
			b_{30}	%	b_{30}	%	b_{30}	%
Tabun	176	30	39.5	77.5	83.5	42.5	128	27
	164	60	71.5	56.5	129	21	113.5	31
DFP	153	30	0	100	0	100	29	81
	148.5	30	4	97	53	64	79.5	46.5

for various lengths of time and subsequently separating the two phases, the cholinesterase inhibiting activity was measured against cobra venom and acetylcholine. It will be seen that certain amounts of Tabun, depending on the length of shaking, go over from the aqueous to the oil phase. Similar observations were made with TEPP.

The inhibition of blood cholinesterases in vivo by Tabun. A series of organophosphorus compounds have been tested for their *in vivo* effects. These experiments were carried out in collaboration with HOLMSTEDT; some of the results obtained have been published (7). Dogs were injected subcutaneously with Tabun and its analogues and blood was taken after various periods of time. No signs of intoxication could be observed after the injection of 0.43 μmol (0.07 mg Tabun) per kg body weight. The *in vivo* cholinesterase inhibiting effect of Tabun (Fig. 9) differs from that of DFP (see review by KOELLE and GILMAN, 33), in reducing the plasma and erythrocyte esterases to the same extent (by the dose given). Many factors other than the esterase inhibition must be considered before such a result can be discussed in detail (see Discussion below). Recent experiments (unpublished) have shown that Tabun and similar organophosphorus compounds react not only with cholinesterases, but with other high and low molecular compounds in the living organism. Until all the complex reactions of these agents *in vivo* are known in detail it will be difficult to give a full explanation to any observation made in such experiments.

Fig. 9 elucidates a typical experiment with Tabun. The initial recovery of the plasma cholinesterase is more rapid than that of the erythrocyte acetylcholinesterase. This probably is due to the comparatively rapid resynthesis of the former enzyme in the liver. Full restoration, however, takes about three weeks (not marked in Fig. 9) for both types of esterase. In the few experiments carried out so far with dogs pretreated with atropine it has been observed that both types of blood esterase are reduced to a smaller extent than in untreated animals (Fig. 9 A). It is known that atropine, in addition to the reaction with the receptor protein at the neuromuscular and synaptic junctions, competes with acetylcholine for the active esterase centre. It is, however, much too early to make any further comment on this point at the present time.

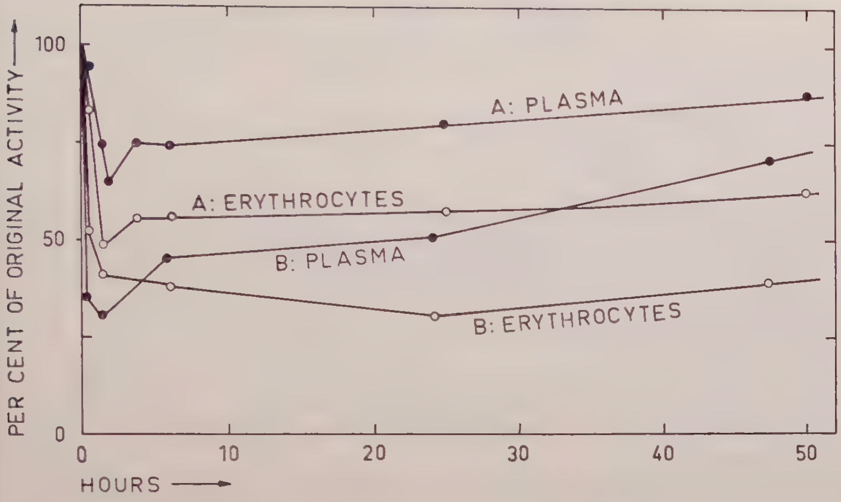

Fig. 9. The effect of Tabun on blood cholinesterases *in vivo* after subcutaneous injection of 0.06 mg Tabun per kg in atropinized (A) and untreated (B) dogs.

Discussion

Tabun or dimethylamido-ethoxy-phosphoryl cyanide is a typical irreversible cholinesterase inhibitor with all the characteristics previously described for other such organophosphorus compounds. For the present we can also explain all the effects of Tabun on different organ systems as being due to inactivation of cholinesterases and the cause of death due to Tabun is similarly explained.

The role played by the various types of acetylcholine splitting enzymes in the toxicological manifestations produced by cholinesterase inhibitors is not clear. On the whole, the physiological significance of the *plasma cholinesterase* is still obscure. Organophosphorus compounds of the "anticholinesterase" type have generally great affinity for this enzyme, the affinity of some compounds is greater than that for the acetylcholinesterases which are believed to play the role of hydrolysing acetylcholine at the sites of its action. It may be that the "transport" plasma cholinesterase (34) is a "barrier" which, in combining with the toxic compound, prevents the more vital enzyme, *i.e.*, the acetylcholinesterase, from being destroyed. The consequence of this might be that compounds found to protect the plasma esterase against inactivation by the toxic compound, give rise to a greater degree of intoxication instead of the expected improvement. In such a case, the protector has to be mixed with atropine, and even with a third compound, as found by GREPPIN (13). However, the most suitable product for preventing symptoms of intoxication after the administration of irreversible cholinesterase inhibitors should be a preparation of plasma cholinesterase.

There are always great difficulties when *in vitro* observations are compared with those *in vivo*. A great many factors must be considered when a complete explanation of one or the other observation is wanted. The following are the factors which are significant in this respect:

A) Factors influencing the cholinesterase inhibition *in vitro:*

1. The affinity of the inhibitor for the enzyme and the reaction mechanism o the inhibiting process.
2. Inhibitor concentration; generally, the inhibition is independent of substrate concentration for irreversible inhibitors.
3. Solvent used for the inhibitor.
4. Enzyme concentration used during incubation with inhibitor.
5. Enzyme source; crude preparations of the same type of enzyme from the same animal or different animals may be found to be attacked differently due probably to one or all of the following reasons:
    a) The inhibitor reacts with constituents in the preparation other than th cholinesterases;
    b) The inhibitor is hydrolysed or otherwise changed by the extract prior t its reaction with the esterases (or other constituents);
    c) Secondary constituents of the preparation may interfere with the reactio mechanism.
6. The sequence of mixing the three components of a complete system: enzyme substrate, and inhibitor.
7. Incubation period, *i.e.*, the length of time during which enzyme and inhibito are in contact with each other before the esterase activity is determined.
8. Temperature and pH of the reaction mixture.

B) Factors influencing the cholinesterase inhibition *in vivo*:

1–8. All factors for *in vitro* inhibition.
9. Distribution in the body via the circulating system.
10. Resorption from blood to tissue.
11. Localization of enzyme in the tissues.
12. Permeability, dependent on the chemical and physical properties of the inhib itor and the morphological and histochemical properties of the tissue mem branes.
13. Physical factors; lipid solubility, etc.
14. Chemical reactions (hydrolysis and others) of the inhibitor before contac with esterase; the cholinesterase inhibiting effect may then be increased (*e.g.* octamethylpyrophosphoramide) or decreased (*e.g.*, DFP).

Summary

A biochemical study, mainly *in vitro*, has been performed with Tabun (dimethyl amido-ethoxy-phosphoryl cyanide) and some allied organophosphorus compound

The acetylcholinesterase inhibiting effect of various organophosphorus compound *in vitro* was tested and expressed in the molar concentration giving 50 per cen inhibition (pI_{50}) (Table 1 and Fig. 1). The principle of the experimental procedur for evaluating I_{50} is illustrated (Fig. 2) for bis(dimethylamido)-phosphoryl fluoride The most active cholinesterase inhibitor studied was dimethylamido-*iso*propoxy phosphoryl cyanide ($pI_{50} = 8.20[8.90]$) which is still more active than Tabu ($pI_{50} = 7.95[8.65]$). The inhibition of cholinesterases by Tabun at various incubatio periods was determined for human plasma and erythrocytes, cat thrombocyte dog nucleus caudatus, electric tissue, and cobra venom (Table 2). The differenc

observed between the inhibition of plasma cholinesterase and that of the acetylcholinesterases (Table 3) is not due to a true selective difference for the two types of esterase, but is the result of a "second" reaction between Tabun and other constituents of the plasma.

The inhibition of acetylcholinesterase by Tabun is dependent on pH in a way similar to that found for the enzyme activity itself (Fig. 3).

The esterase inhibition (inactivation) by Tabun is an irreversible process (Fig. 4).

Various groups of compounds have been tested as protectors of cholinesterases against inactivation by Tabun (in preliminary experiment by TEPP) (Tables 4 and 5). Those particularly studied are acetylcholine (Fig. 5), physostigmine and prostigmine (Fig. 6), and the following three compounds, the properties of which as esterase inhibitors have been exhaustively investigated: procaine, pantocaine, and parpanit (Fig. 7).

Tabun reacts spontaneously with adrenaline and other dihydroxyphenyl derivatives (Table 6). This reaction is considered to be a phosphorylation at one of the phenol groups.

The stability of aqueous solutions of Tabun (Fig. 8), and the distribution of Tabun between water and peanut oil (Table 7) are reported briefly.

The *in vivo* effect of Tabun on blood cholinesterases after subcutaneous injection of Tabun in atropinized and untreated dogs is demonstrated (Fig. 9).

The general factors which have to be considered in biochemical studies with the toxic organophosphorus compounds are discussed.

It is a great pleasure to have this opportunity of expressing my gratitude to Professor Hilding Berglund, Professor Gustaf Ljunggren, Professor Torsten Teorell, and Dr. Carl-Johan Clemedson for their continuous interest in this investigation. To Dr. Bo Holmstedt I beg to acknowledge my indebtedness for stimulating collaboration in the early part of the present investigation. The various organophosphorus compounds were prepared at the Research Institute of National Defence, Department 1, Chief Professor Gustaf Ljunggren.

For skilful technical assistance I am greatly indebted to Mrs Margareta Grahn, Miss Jenny Holm, Miss Inger Söhrman, and Mrs Britta Tyrefors.

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Note added in proof: Most recently (April 3, 1953), the first account on the use of Tabun and allied compounds as insecticides has been released for publication. It is in the German patent 767, 723, with Dr. Gerhard Schrader and Dr. Ing. Hans Kükenthal as inventors. This document is of historical interest, as it may be regarded as the very first announcement on Tabun; the invention was patented in Germany Febr. 3, 1937, even before the synthesis of the compound was published (cf. ref. 5).

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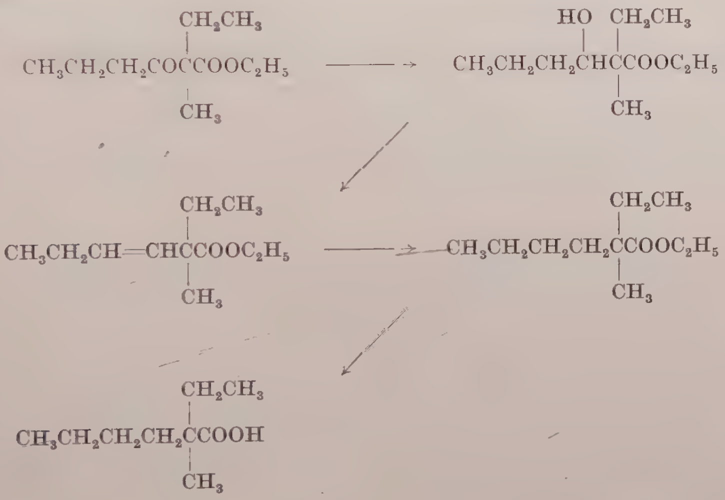
A method for the preparation of trialkylacetic acids

By ARNE BRÄNDSTRÖM and INGEMAR FORSBLAD

Many methods have been tried to obtain trialkylacetic acids (1) but none has been entirely satisfactory for the preparation of these acids in quantity. In the present investigation, *α-methyl-α-ethylhexanoic acid* has been prepared by the following steps:



$$CH_3CH_2CH_2CO\underset{CH_3}{\overset{CH_2CH_3}{C}}COOC_2H_5 \longrightarrow CH_3CH_2CH_2\overset{HO}{C}H\underset{CH_3}{\overset{CH_2CH_3}{C}}COOC_2H_5$$

$$\longrightarrow CH_3CH_2CH{=}CH\underset{CH_3}{\overset{CH_2CH_3}{C}}COOC_2H_5 \longrightarrow CH_3CH_2CH_2CH_2\underset{CH_3}{\overset{CH_2CH_3}{C}}COOC_2H_5$$

$$\longrightarrow CH_3CH_2CH_2CH_2\underset{CH_3}{\overset{CH_2CH_3}{C}}COOH$$

As dialkylated β-ketoesters are readily available starting materials and the yields good, this method is probably often the most convenient one for the preparation of trialkylacetic acids.

In order to demonstrate that no rearrangements occurred, the same acid was also prepared by carbonisation of the appropriate Grignard reagent. The acids prepared by these two methods were shown to be identical by means of their *benzyl-isothiuronium salts*.

Experimental

Ethyl α-methyl-α-ethyl-β-oxohexanoate

In a 2-litre, three-necked, round-bottomed flask fitted with a reflux condenser, a sealed stirrer and a dropping funnel, 31.5 g of sodium were dissolved in 600 ml of absolute alcohol (distilled from sodium and ethyl phthalate). When all the sodium had dissolved, 250 g of ethyl *n*-butyryl-*n*-butyrate were added, followed by 200 g of methyl iodide. The mixture was refluxed for three hours, 20 g of methyl iodide were then added, and the refluxing continued for three hours more. The alcohol was distilled from the resulting neutral mixture and the residue shaken with water to dissolve the sodium iodide. The ester layer was separated and the water layer extracted with benzene. The combined ester and benzene portions were washed with two 200 ml portions of 5 per cent potassium hydroxide in order to remove any unchanged ethyl *n*-butyryl-*n*-butyrate. After a final washing with water the solution was dried over anhydrous sodium sulphate, the benzene removed by distillation, and the residue fractionated under reduced pressure with the use of a Widmer column. The fraction boiling at 97–100°/8 mm was collected, and amounted to 217 g, or 81 per cent of the theoretical amount.

The product did not give any enol reaction when tested with a ferric chloride solution.

Ethyl α-methyl-α-ethyl-β-oxyhexanoate

215 g of the ketoester above were hydrogenated at 185° and 150 atm using 10 g of Raney nickel as catalyst until no more hydrogen was absorbed (three hours). No solvent was used. After cooling the catalyst was removed by filtration and washed with alcohol. The resulting liquid was distilled under reduced pressure and the fraction boiling at 106–108°/8 mm collected. The yield was 201 g or 93 per cent of the theoretical amount.

This oxyester was markedly more viscous than the corresponding ketoester.

Ethyl α-methyl-α-ethyl-β, γ-hexenoate

200 g of the ester above were dissolved in 600 ml of benzene and refluxed with 180 g of phosphorous pentoxide for three hours. The benzene layer was decanted off and the residue extracted by refluxing with fresh benzene. The combined benzene layers were shaken once with water. The residue in the flask was dissolved in water and the solution extracted twice with ether. The combined ether portions were washed with water, combined with the benzene portions and dried over anhydrous sodium sulphate. The solvent was removed by distillation, and the residue fractionated under reduced pressure using a Widmer column. The yield was 118 g (65 per cent of the theoretical amount) of a product boiling at 78–81°/8 mm. 23 g of the oxyester were recovered.

Ethyl α-methyl-α-ethylhexanoate

118 g of the unsaturated ester above were hydrogenated at 150° and 150 atm using 5 g of Raney nickel as catalyst. No solvent was used. When no more hydrogen was absorbed (one hour) the catalyst was removed by filtration and washed with alcohol. The resulting filtrate was distilled under reduced pressure yielding 104 g of a product boiling at 76–78°/8 mm. This is 87 per cent of the theoretical amount.

α-Methyl-α-ethylhexanoic acid

The ester above is rather difficult to hydrolyse by the usual methods, but the use of a mixture of cellosolve and xylene offers a suitable reaction medium (2) in which this ester is readily hydrolysed. Owing to a discolouration of the product as a result of air oxidation of the basic solution, it was necessary to carry out all the following operations under nitrogen. The colour produced without this precaution was carried over to the acid and could not be removed by distillation.

200 ml of xylene and 200 ml of cellosolve were refluxed for 1 hour with 10 g of potassium hydroxide. The solvents were then distilled into a 1-litre round-bottomed flask. 80 g of potassium hydroxide were dissolved in the solvent, the ester added and the mixture heated to about 120° for one hour in an apparatus arranged for downward distillation. Most of the solvent was then distilled off. The residue was allowed to cool, diluted with 500 ml of water and the solution extracted with ether. This ether solution contained 15 g of unchanged ester.

The water solution was acidified with conc. hydrochloric acid and the acid extracted with benzene. The benzene solution was dried with anhydrous sodium sulphate, the solvent removed by distillation, and the residue fractionated under reduced pressure yielding 70 g (80 per cent of the theoretical amount) of a water-white acid boiling at 121–122°/10 mm.

Anal. Subst. 48.35 mg: 4.448 ml of 0.06847-N NaOH.
» » 14.66 mg: 36.73 mg CO_2, 15.12 mg H_2O.
$C_9H_{18}O_2$ (158.23) calc. Equ. wt. 158.23; C 68.31; H 11.46.
found Equ. wt. 158.68; C 68.35; H 11.54.

The *benzyl*-iso-*thiuronium salt* of the acid melted at 117.5–118°.

α-Methyl-α-ethyl-n-*amyl alcohol* was prepared by the method of F. C. WHITMORE and D. E. BADERTSCHER (3). The yield of the alcohol boiling at 58–60°/8 mm was 70 per cent of the theoretical amount.

α-Methyl-α-ethyl-n-*amyl chloride* was prepared by the same authors' method (3). The yield of the chloride boiling at 51–52°/10 mm was 87 per cent of the theoretical amount.

α-Methyl-α-ethylhexanoic acid was obtained by adding CO_2 at —3° to *α-methyl-α-ethylamylmagnesium chloride* prepared as described by the above authors (3). The product was isolated by a standard method (4) yielding 6 per cent of the theoretical amount of the acid boiling at 119.5–120.5°/8 mm.

The benzyl-iso-thiuronium salt of this acid melted at 117.5–118°, and showed no melting point depression when mixed with the salt of the acid prepared by the other method.

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Tryckt den 10 september 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 33

Communicated 3 June 1953 by Arne Fredga

Stereochemical studies on plant growth regulators. VI[1]

The steric connections between optically active α-aryloxyalkylcarboxylic acids, lactic acid and α-alanine

By Magnus Matell

With 1 figure in the text

In earlier papers the establishment of the steric relations between several optically active plant growth regulators of the α-aryloxyalkylcarboxylic acid type have been described (1–5). Considering their biological effects (6–8), it was found that these compounds show stereochemical specificity as regards their plant growth regulating properties. For a further understanding of the growth regulating mechanism, it is important to connect them sterically to the glyceric aldehyde system. Experiments with such an end in view have already been reported by Fredga and Matell (1, 2) who conclude, that acids with greater auxin activity than their respective antipodes *probably* have the D-configuration. The uncertainty involved in this result is small, but a verification, utilising reactions without substitution at the asymmetric carbon atom, seemed to be important.

The optically active α-phenoxypropionic acid was sterically connected to lactic acid in the following way. The sodium compound of ethyl lactate, containing excess of the L-(−)-form, was treated with 2.4-dinitrochlorobenzene (DNCB) in abs. ether. This gave a dextrorotatory ester, which on acid hydrolysis yielded α-(2.4-dinitrophenoxy)-propionic acid, containing excess of the (+)-form. (+)-α-Phenoxypropionic acid on nitration yielded (—)-α-(2.4-dinitrophenoxy)-propionic acid and consequently has the same configuration as ethyl D-(+)-lactate (Fig. 1). The steric relations between α-phenoxypropionic acid and α-alanine were established as follows. L-(+)-α-Alanine was reacted with DNCB yielding (+)-N-(2.4-dinitrophenyl)-α-alanine, which was found to give a quasi-racemic compound with (—)-α-(2.4-dinitrophenoxy)-propionic acid (Fig. 1, Table 2). Thus the two last-mentioned compounds have opposite configurations and consequently (+)-α-phenoxypropionic acid has the configuration opposite to that of L-(+)-alanine, *viz.* D (Fig. 1).

As both the results agree and furthermore are in accordance with previous experiments, it may be definetely stated that (+)-α-phenoxypropionic acid belongs to the D-series; it is represented by the stereoformula I. According to previous works (1–8) those active α-aryloxyalkylcarboxylic acids, which have greater auxin activity than their respective antipodes, have the same configuration as (+)-α-phenoxypro-

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[1] Part V: Arkiv Kemi, *6*, 251 (1953).

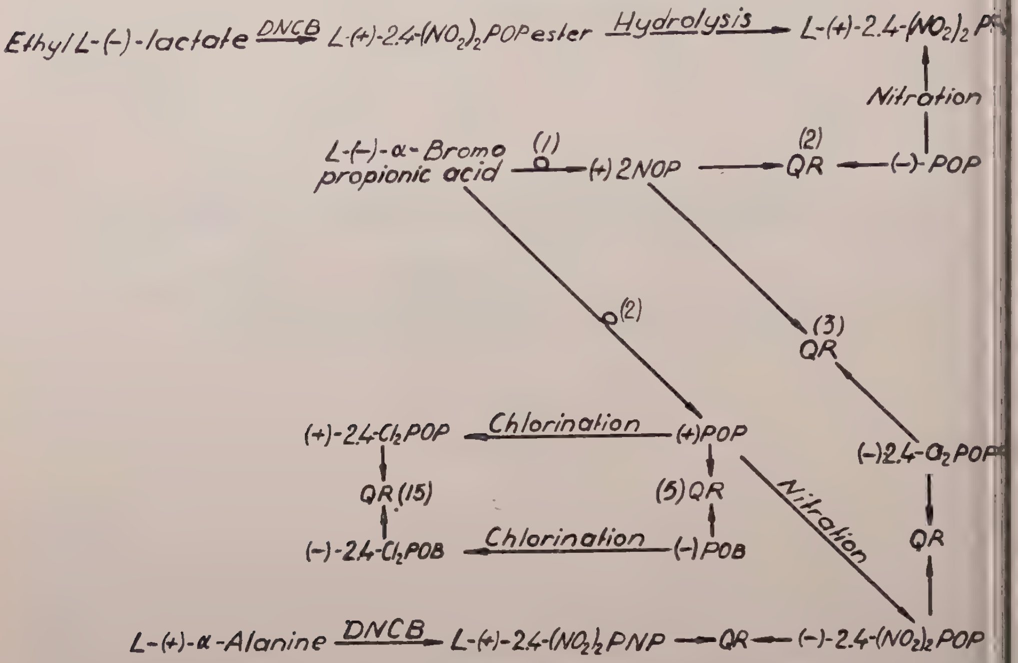
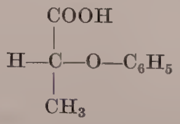

Ethyl L-(-)-lactate
DNCB
L-(+)-2.4-(NO2)2POP ester
Hydrolysis
L-(+)-2.4-(NO2)2 P
Nitration
L-(-)-α-Bromo propionic acid
(1)
(+) 2NOP
(2)
QR
(-)-POP
(2)
(3)
QR
(+)-2.4-Cl2POP
Chlorination
(+)POP
(-)2.4-O2POP
QR (15)
(5)QR
Nitration
(-)-2.4-Cl2POB
Chlorination
(-)POB
QR
L-(+)-α-Alanine
DNCB
L-(+)-2.4-(NO2)2PNP
QR
(-)-2.4-(NO2)2POP

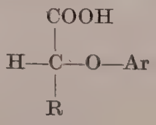
Fig. 1. The establishment of the steric relations of α-aryloxyalkylcarboxylic acids. References are given by numbers in parentheses. QR means that compounds from which arrows are coming form a quasi-racemic compound. α-Phenoxypropionic acid and α-phenoxy-*n*-butyric acid are abbreviated POP and POB respectively. PNP means N-phenyl-α-alanine. The other abbreviations are self-evident, *e.g.* 2.4-$(NO_2)_2$POP means α-(2.4-dinitrophenoxy)-propionic acid.

pionic acid. Thus they have the D-configuration and are represented by the general formula II (R stands for an alkyl group and Ar for an aromatic rest).



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H—C—O—C_6H_5} \\ | \\ \mathrm{CH_3} \end{array}$$

I



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H—C—O—Ar} \\ | \\ \mathrm{R} \end{array}$$

II

In conjunction with the above-mentioned experiments some other steric relations have been verified. Optically active α-phenoxypropionic acid and α-phenoxy-*n*-butyric acid were chlorinated to the corresponding 2.4-dichloro compounds. In both cases these showed the same direction of optical rotation as the starting materials. The phenoxy- and 2.4-dichlorophenoxy acids with the same direction of optical rotation thus have the same configuration. The same result has been obtained previously, (3, 5, 9) using the method of quasi-racemates (10, 11) which must be regarded as being very reliable. However, this method is founded partly on inference by analogy, partly on empiric results, and therefore the results obtained by this method should be confirmed in other ways when possible.

Finally it was found that α-(2.4-dinitrophenoxy)-propionic acid and α-(2.4-

dichlorophenoxy)-propionic acid with the same direction of rotation form a quasi-racemic compound (Table 2) and consequently have opposite configurations. This result too is in accordance with previous results (Fig. 1).

Experimental

α-(2.4-Dinitrophenoxy)-propionic acid. *a) From optically inactive ethyl lactate.* To finely cut sodium (2.3 g, 0.1 g atom) covered with abs. ether and cooled in ice was added ethyl lactate (13 g, 0.11 mole) dissolved in 25 ml of abs. ether. The addition of the lactate solution took 4 hours and after further 2 hours all the sodium had reacted. 2.4-Dinitrochlorobenzene (DNCB) (20.3 g, 0.1 mole) dissolved in 50 ml of abs. ether was slowly added (1 hour) to the ice cooled solution. The solution became dark reddish brown when the DNCB was added and a precipitate was immediately formed. The reaction was completed by refluxing for 2 hours on a steam-bath. The product was treated with water and the ethereal layer separated and dried over sodium sulphate. The ether was driven off and the remaining viscous oil, which crystallised within a few days, was distilled *in vacuo.* The lower boiling fractions contained unreacted ethyl lactate and DNCB; of the latter 9.3 g (46 %) were recovered. The last fraction, boiling at 190–198°/2 mm, soon crystallised. The yellow, crystalline product weighed 12.7 g (45%) and melted at 64–69°.

3.0 g ethyl α-(2.4-dinitrophenoxy)-propionate was refluxed for 2 hours with 20 ml dilute (1:1) hydrochloric acid. On cooling the organic acid crystallised as long, yellow needles. The crude acid (2.0 g, 74%) was recrystallised from dilute formic acid. M. p. 171–173.5°.

114.73 mg acid: 9.015 ml 0.04955-*N* NaOH
$C_9H_8O_7N_2$ calc. equiv. wt. 256.2
found » » 256.8

b) From optically active ethyl lactate. Starting with ethyl lactate with $[\alpha]_D^{20} = -1.19°$ (homogeneous) an ester boiling at 190–198°/2 mm was obtained in 38% yield. The procedure was the same as described above. It melted at 64–68° and was slightly dextrorotating; $[\alpha]_D^{20} = +2.8°$ ($c = 2.9$ in abs. alcohol).

When hydrolysed with hydrochloric acid as described above an acid with m.p. 160–169° was obtained. The large melting point interval is probably due to the fact that the product is a mixture of the racemic acid and the (+)-form. When mixed with about an equal quantity of the pure, inactive acid, it melted at 162–171°.

89.48 mg acid: 7.005 ml 0.04955-*N* NaOH
$C_9H_8O_7N_2$ calc. equiv. wt. 256.2
found » » 257.8

0.2967 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{20} = +0.035° \pm 0.003$; $[\alpha]_D^{20} = +1.2° \pm 0.1°$.

The optical activity of the product as compared with that of the optically pure acid (*vide infra*) is much lower than could be expected from the activity of the raw material. This may depend on partial racemization due to the high boiling point of the ester and loss of activity in the recrystallisation of the acid. Therefore in another experiment, the crude ester was hydrolysed without further purification and the acid was not recrystallised, as it was found that this procedure lowered the activity considerably. However, the intense colour of the crude acid made the optical activity measurement difficult with the result that the standard error is rather large (±0.012°) as compared with that of the measurement of the purified compound (±0.003°).

0.2316 g crude acid in abs. *alcohol* to 10.00 ml: $\alpha_D^{20} = +0.111° \pm 0.012°$; $[\alpha]_D^{20} = +4.8° \pm 0.5°$.

This value corresponds to 47.7% (−)-acid ($[\alpha]_D^{22} = -105.1°$, $c = 2.0$ in abs. alcohol) while the ethyl lactate used contained 45.9% D-(+)-form, if it is assumed that the optically pure ethyl lactate has $[\alpha]_D^{19} = 14.52°$ (12). Thus in this latter experiment less than half the activity was lost.

c) From α-phenoxypropionic acid. α-Phenoxypropionic acid (2.5 g) was slowly added to white fuming nitric acid (8.2 ml, sp. gr. 1.50) cooled in ice. The mixture was poured into water (50 ml) and the crystalline product collected. The crude acid (3.21 g, 82%) which melted at 171–173.5° was recrystallised from 45% formic acid. The pure acid melted at 171.5–174°. When mixed with α-(2.4-dinitrophenoxy)-propionic acid, prepared according to procedure a) above, it melted at 171–174° and the identity of the preparations therefore seems to be established.

100.15 mg acid: 7.925 ml 0.04955-*N* NaOH

$C_9H_8O_7N_2$ calc. equiv. wt. 256.2

found » » 255.0

d) From (+)-α-phenoxypropionic acid. (+)-α-Phenoxypropionic acid (0.44 g) with $[\alpha]_D^{20} = +40.1°$ ($c = 2.0$ in abs. alcohol) was treated with nitric acid as described in c). 0.55 g (81%) crude acid was collected. M.p. 140–143.5°. Recrystallisation from 45% formic acid yielded 0.27 g pure acid with m.p. 142–143.5°.

94.17 mg acid: 7.435 ml 0.04955-*N* NaOH

$C_9H_8O_7N_2$ calc. equiv. wt. 256.2

found » » 255.6

0.1671 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{22} = -1.757°$. $[\alpha]_D^{22} = -105.1°$; $[M]_D^{22} = -269.4°$.

N-(2.4-Dinitrophenyl)-α-alanines. These compounds were prepared from 2.4-dinitrochlorobenzene and *rac.*-α-alanine, D-(−)-α-alanine and L-(+)-α-alanine respectively according to ABDERHALDEN and BLUMBERG (13). The following melting points and optical activities were found (Table 1).

Table 1. Physical constants of substituted alanines.[1]

Compound	Recryst. from	M.p.	$[\alpha]_D^{22}$
rac.-2.4-$(NO_2)_2$PNP	50 % formic acid	175–177° (d)	—
D-(−)- »	50 % ethanol	174–178° (d)	−6.5° (c = 1.1 in abs. alcohol)
L-(+)- »	50 % »	175–178° (d)	+6.9° (c = 0.9 » » »)

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[1] For abbreviations, see Fig. 1.

α-2.4-Dichlorophenoxyalkylcarboxylic acids. The general procedure given by HASKELBERG was followed (14).

A solution of *α-phenoxypropionic acid* (1.66 g, 0.01 mole) in glacial acetic acid (4 ml), containing a trace of iodine was chlorinated at 30° until the weight had increased 1.75 g. The product was poured into water when an oil, which crystallised in a short time, separated. The crude product (2.10 g, 89%) was recrystallised from petroleum (b.p. 60–80°). The pure acid (1.15 g) melted at 115.5–116.5°. When mixed with a sample of α-(2.4-dichlorophenoxy)-propionic acid (m.p. 116–117°), it melted at 114.5–116°.

In a similar way (−)-*α-phenoxypropionic acid* was chlorinated. The product was recrystallised from petroleum (b.p. 60–80°). M.p. 120.5–122.5°. A mixture of this compound and a sample of pure (−)-α-(2.4-dichlorophenoxy)-propionic acid (M.p. 122.5–123°, $[\alpha]_D^{25} = -35.2°$, $c = 1.0$ in acetone) melted at 121–123°. Thermal analysis of the product and (+)-α-(2.4-dichlorophenoxy)-propionic acid by the contact method (16) showed a molecular compound, m.p. 117°, and two eutectics at about 110° (Table 2). These datas are in good accordance with the melting point diagram of the same enantiomorphic acids (Table 2). Finally the optical activity is in excellent agreement with the value given elsewhere (15). Thus there is no doubt that the product is identical with (−)-α-(2.4-dichlorophenoxy)-propionic acid.

0.1775 g dissolved in *acetone* to 20.06 ml: $2\alpha_D^{25} = -0.625°$. $[\alpha]_D^{25} = -35.3°$.

α-Phenoxy-n-butyric acid was chlorinated at room temperature with a trace of iron as catalyst. The product was recrystallised from petroleum (b.p. 60–80°) and was found to be dimorphic; the less stable modification melted at 79–82° and the more stable one at 84.5°. This is in excellent agreement with the data found for α-(2.4-dichlorophenoxy)-*n*-butyric acid (9), which melted at 80–82° and 84.5°. A mixture of the two compounds melted at 79–84.5°.

When (+)-*α-phenoxy-n-butyric acid* was treated with chlorine in the same way a dextrorotating acid was obtained. M.p. 89–91°.

0.0678 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{23} = +0.283°$. $[\alpha]_D^{23} = +41.7°$.

Table 2. Thermal data for some two-component systems[1]

Component 1	Component 2	Eutectics	Mol. compound. M.p.	Notes
(+)-2.4-Cl_2POP	Chlorinated (−)-POP	110°, 110°	117°	
(+)- »	(−)-2.4-Cl_2POP	109°, 109°	118°	(3)
(−)-2.4-Cl_2POB	Chlorinated (+)-POB	75.5°, 75.5°	81.5°	
(−)- »	(+)-2.4-Cl_2POB	75.5°, 75.5°	82°	(9)
(+)-2.4-$(NO_2)_2$PNP	(−)-2.4-$(NO_2)_2$POP	160°, 139.5°	167°	
(−)- »	(−)- »	—	—	Type I[2]
(−)-2.4-Cl_2POP	(−)- »	119.5°, 131°	136°	—
(+)- »	(−)- »	107°	—	V[2]

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[1] For abbreviations see Fig. 1.

[2] According to ROOZEBOOM.

The data for (+)-α-(2.4-dichlorophenoxy)-*n*-butyric acid are: M.p. 91–91.6°; $[\alpha]_D^{25} = +41.8°$ ($c = 2.1$ in abs. alcohol). Further proof of the identity of the compounds was obtained by thermal analysis of the product and (−)-α-(2.4-dichlorophenoxy)-*n*-butyric acid by the contact method (16). The components formed a molecular compound melting at 81.5° and two eutectics at 75.5° (Table 2). The melting point diagram of the enantiomorphic α-phenoxy-*n*-butyric acids show a molecular compound, m.p. 82°, and two eutectics at 75.5° (Table 2).

Thermal analysis. Contact preparations were studied in a Kofler hot stage microscope (16). The results are found in Table 2. The same apparatus was used for all melting point determinations.

The author is much indebted to Prof. A. Fredga for valuable discussions as well as for the supply of some of the starting materials. Thanks are due to Prof. N. Hellström, the head of this department, for his kind interest in this work and for all facilities put at the author's disposal. The author is indebted to Mr. E. Jonsson for technical assistance. Part of the expenses involved have been defrayed by a grant from *Statens Naturvetenskapliga Forskningsråd*, which is gratefully acknowledged.

SUMMARY

(+)-α-Phenoxypropionic acid has been shown to have the same configuration as D-(−)-α-alanine and ethyl D(+)-lactate (Fig. 1). As a consequence of this, it is proved that optically active α-aryloxyalkylcarboxylic acids, which have greater auxin activity than their respective antipodes, have the D-configuration.

Optically active α-phenoxypropionic acid and α-phenoxy-*n*-butyric acid have been chlorinated yielding the corresponding 2.4-dichlorophenoxy compounds, which were found to have the same direction of optical rotation as the starting materials. The steric relations established in this way are in full agreement with earlier results obtained by the method of quasi-racemates (Fig. 1).

Department of Organic Chemistry, Royal Agricultural College, Uppsala, May 1953.

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Vorgelegt am 13. Mai 1953

Einflüsse chemischer Faktoren auf die Entwicklung von Tumoren. I

Von HANS VON EULER und BETH VON EULER

Naphtochinon-methid und Tetraphenyl-fulven

Die positive oder negative Entwicklung (Wachstum oder Regression) von Cancerzellen wird nach den früheren Ergebnissen dieses Institutes[1] von den als oxydoreduktive Katalysatoren wirksamen Enzymsystemen stark beeinflusst, und zwar handelt es sich dabei in erster Linie um Eisen-porphyrine, besonders Cytochrom-Systeme incl. deren Aktivatoren sowie um deren Antagonisten, nämlich blockierende Stoffe und Reduktionsmittel, besonders SH-haltige Substanzen.

Der Ausgang des Kampfes zwischen dem normalen und dem cancerösen Gewebe hängt wesentlich davon ab, wie sich die oxydativen Einflüsse auf diese beiden Gewebe-Typen verteilen.

Es muss übrigens hier gleich betont werden, dass Oxydationsmittel wie Chinone auch als Hemmungsstoffe von Enzymen fungieren können, und dass andererseits p- bezw. p,p'-Dioxyderivate des Benzols und Diphenyls (Hydrochinon, p-Chinon, 4,4'-Dioxystilben[2] sowie entsprechende Naphtalinderivate) sich als besonders wirksame tumorvernichtende bezw. mitosehemmende Stoffe erwiesen haben.

Bezüglich der Chinone ist zunächst folgendes zu bemerken: N. TAKIZAWA[3] wies zuerst *cancerogene* Wirkungen von p-Benzochinon nach. In Bestätigung der Arbeiten von KENSLER u. Mitarb.[4] fanden R. KUHN und BEINERT[5], dass die Wirkung der Wursterschen Farbsalze auf die Oxydationsprodukte der Diamine zurückzuführen ist und betonen die starke Hemmung des p-Benzochinons auf die Hefencarboxylase.

Nach E. F. LEHMANN's[6] (am Ringelwurm, *Tubifex*, geführten) Nachweis des antimitotischen Effektes von Chinonen sind Wirkungen derselben auf die Tumorentwicklung oft, und zwar mit recht verschiedenen Ergebnissen untersucht worden. Sehr wahrscheinlich spielt hierbei die Verteilung der Chinone und Chinonderivate auf die noch normalen und die cancerösen Gewebe eines vom Krebs befallenen Organismus eine wesentliche Rolle. Kein anderes Anti-mitotikum (kein Chinon) is übrigens so wirksam wie Colchicin (siehe hierzu auch H. LETTRÉ[1]).

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[1] Siehe besonders EULER u. HELLSTRÖM, Z. physiol. Chem. 260, 163; 1939. — BETH v. EULER u. H. v. EULER, Dieses Arkiv 2, nr 38; 1950. — Siehe auch EULER (Kem. Arb. 14, II, Jan. 1951) sowie die bemerkenswerten Arbeiten von WARO NAKAHARA über Toxohormone (Gann, 40–44; 1949–53).

[2] Siehe H. DRUCKREY, P. DANNEBERG u. D. SCHMÄHL, Naturwiss. 39, 381 u. 393; 1952.

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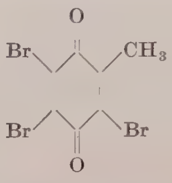
[6] E. F. LEHMANN, Experientia 3, 223; 1947.

Die direkte Wirkung verschiedener Chinone auf Eisen-porphyrine wird hier untersucht.

Tribrom-2-methyl-p-chinon, das wir als Nebenprodukt bei der Darstellung de entsprechenden Tribrom-chinon-methides erhielten, wurde in Tagesdosen von 2 mg in Jensensarkom-tragende Ratten subkutan injiziert, und zwar besonders deshalb weil es sehr leicht Wasserstoff aufnimmt und also als starkes Oxydationsmittel in Betracht kam.



Zum weiteren Vergleich mit früheren Ergebnissen[2] wurden zwei Versuchsreihe angesetzt mit 4,4′-Dioxy-diphenyl und mit dessen Chinon. Über die Einwirkung dieser Stoffe auf die Entwicklung von Jensensarkomen kann erst nach Abschlus der Versuche berichtet werden.[3]

Im Folgenden werden nun zwei spezifische Wirkungsstoffe, Napthochinonmethid und Tetraphenyl-fulven besprochen.

2-Naphtochinon-1-methid. Dieses stabile Produkt wurde nach FRIES u. HÜBNER dargestellt (vgl. EULER u. HASSELQUIST, Dieses Arkiv 6 nr. 12; 1953).

Die Ratten (unser norwegischer, weisser Stamm) erhielten jeden zweiten Tag 2 mg Subst. in Arachidöl gelöst, im Ganzen 13 mg, davon 5 mg subkutan injiziert, 8 mg per os. Die Entwicklung der *Jensen* Tumoren wurde verfolgt durch Palpieren nach den 3 Dimensionen, woraus das Volumen berechnet wurde. Diese Messungen ergaben: für das Gesamtvolumen der Tumoren aller Versuchstiere und Kontrollen

	10 Tage nach Impfung	18 Tage nach Impfung
Versuchstiere	34 ccm	322 ccm
Kontrolltiere	18 ccm	24 ccm

Sämmtliche Tumoren der Versuchstiere wuchsen stark. Der Tumor, von welchem die Impfung vorgenommen wurde, war ungewöhnlich wenig lebenskräftig, hat sich aber in den Versuchstieren auf Grund des stark stimulierenden Effektes des Naphtochinonmethides stark entwickelt.

Wiederholung des Versuchs am 9.–27. Jan. 1953: Erste Injektion am Tage vor der Impfung. Die Dosen waren diesmal kleiner, nämlich 1–2 mg, im Ganzen während des Versuchs 11 mg, die subkutan injiziert wurden.

	12 Tage nach Impfung	17 Tage nach Impfung
Versuchstiere	41,3 ccm	203 ccm
Kontrollen	40,0 ccm	105 ccm

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[1] H. LETTRÉ, Scientia Pharmaceutica, 20, 75; 1950.

[2] An Sarkomen und in dem damit oft parallel gehenden Kressen-Keim-Test.

[3] Erinnert sei an die Oxydation von Pyrogallol zu Purpurogallin (WILLSTÄTTER).

Am Tag nach der letzten Injektion, 22. Jan., hatten die Versuchstiere 4 mittelgrosse und 2 kleine Tumoren, die Kontrollen 3 mittelgrosse und 3 kleine Tumoren.

Nach weiteren 5 Tagen betrugen diese Grössen: Versuchstiere 4 sehr grosse Tumoren und 2 mittelgrosse Tumoren, Kontrollen 2 grosse Tumoren und 4 mittelgrosse Tumoren.

Einen gewissen stimulierenden obwohl kleineren Effekt ergab also auch die zweite Versuchsserie.

Dieses Ergebnis veranlasste:

a) eine dritte Serie mit Jensentumoren tragenden Ratten, 23. März—11. April 1953. Gesamtdosis 15 mg Naphtochinonmethid. 7 Versuchstiere, 5 Kontrollen. Berechnung bei dieser Serie als mittleres Volumen per Tier

	12 Tage nach Impfung	18 Tage nach Impfung
Versuchstiere	18,4 ccm	45,6 ccm
Kontrollen	13,3 ccm	39,7 ccm

Das Resultat ist beeinträchtigt dadurch, dass unter den Versuchstieren eine starke Tumor-regression am 7. Tag nach Impfung festgestellt wurde.

b) eine Versuchsserie mit 10 jungen frischen (nicht cancerösen) Ratten gleichen Stammes 5. März.—2. April 1953. Von diesen erhielten 5 Ratten Naphtochinonmethid, und zwar 5 mal in der Woche je 1 mg in 0,1 ml Arachidöl subkutan injiziert.

	Anfangsgewicht	Schlussgewicht	Differenz
5 Versuchstiere.....	192 g	597 g	405 g
5 Kontrolltiere	190 g	605 g	415 g

An den *normalen* Jungen zeigte das Naphtochinonmethid also *keinen Wachstumstimulierenden Effekt.*

Von den 3 Serien mit Jensensarkom-Ratten zeigte die erste den grössten Einfluss des Chinonmethides. Bei der dritten Serie trat ein gewisser Ausgleich der Zuwachsgeschwindigkeit der beiden Gruppen während der 3. Woche nach der Injektion ein.

Immerhin erscheint uns die Wachstumswirkung des Naphtochinon-methides so deutlich, dass weitere Versuchsserien in Aussicht genommen sind.

2,3,4,5-Tetraphenyl-fulven. Dieses stabile Fulven wurde dargestellt aus 2,3,4,5-Tetraphenyl-1-methyl-cyclopentadien-1-ol durch eine Modifikation der von DILTHEY und HUCHTEMANN[1] gegebenen Vorschrift, wobei statt HCl von H. HASSELQUIST Schwefelsäure verwendet wurde. Das verwendete Präparat wurde in Form roter Nadeln vom Schmpt. 210–211° erhalten und kam als Öl-Suspension sowohl bei der subkutanen Injektion als bei der Eingabe per os zur Verwendung.

Erste Injektion 1 Tag vor der Impfung, letzte Injektion 15 Tage nach der Impfung. Tagesdose 2 mg, Gesamtdose 26 mg per Ratte.

12 Tage nach der Impfung wurde die Tumoren palpiert und wie S. 362 beschrieben gemessen

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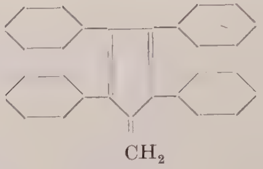
[1] DILTHEY und HUCHTEMANN, J. prakt. Chem. 154, 250; 1940.



Das Resultat dieser 1. Serie ergibt sich aus folgenden Zahlen[1]:

	12 Tage nach Impfung	18 Tage nach Impfung
6 Versuchstiere	62,9 ccm	208 ccm, 2 retrograd
6 Kontrollen	85,9 ccm	379 ccm, alle wachsend

Beobachtung über eine Pelz-Anomalie bei jungen Ratten

Am 22. März 1953 betrug der Wurf eines Ratten-Weibchens 12 Junge, von welchen man ihr 5 überliess. Das Weibchen erhielt ausserdem 4 fremde Jungen des gleichen Stammes, die es gleichzeitig mit den eigenen Jungen ernährte.

Die 5 eigenen Jungen waren nach 21 Tagen entwöhnt, die fremden Jungen nach 24 Tagen. Schon 2 Tage bevor die eigenen Jungen entfernt wurden, wurde am Weibchen schleimiges Erbrechen beobachtet. Es musste getötet werden 2 Tage nach Entfernung der fremden Jungen. Bei der Obduktion zeigte es sich, dass Leber und Magen zusammengewachsen waren, die Leber zeigte grosse Abscesse. Das Peritoneum war in die pathologischen Veränderungen einbezogen, auch die Umgebung der Milz. Deutlich war die Veränderung der *Leber.*

Alle Jungen, sowohl die eigenen wie die fremden, erschienen lebhaft und frisch. Bei allen war aber der Pelz dünn behaart. Die Schultern und die Mitte des Rückens waren nur von wenig Flaum bedeckt. Diese Erscheinung wurde deutlich eine Woche nach der Entwöhnung, als die gleichalterigen Tiere dichten und gleichmässigen Pelz trugen. Das Symptom nahm allmählich ab, besonders rasch und vollständig bei 2 Tieren, die mit Eingeweiden und Fett von Huhn und mit Käse ernährt wurde. Es lag allem Anschein nach bei den pelzarmen Tieren eine durch die anomale Muttermilch hervorgerufene Avitaminose vor.

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[1] In einer 2. Serie (12 Tiere) zeigte sich dagegen keinerlei Hemmung.

Tryckt den 10 september 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

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Communicated 3 June 1953 by ARNE FREDGA

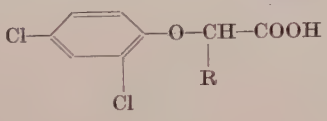
Stereochemical studies on plant growth regulators. VII[1]

Optically active α-(2-methyl-4-chlorophenoxy)-propionic acid and α-(2.4-dichlorophenoxy)-*n*-butyric acid and their steric relations

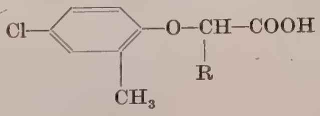
By MAGNUS MATELL

With 6 figures in the text

2.4-Dichlorophenoxyacetic acid (I, R = H) and 2-methyl-4-chlorophenoxyacetic acid (II, R = H) have proved to be very valuable in practical agriculture and so their plant growth regulating properties have been extensively studied. Several homologous acids have been synthesised and tested biologically, but up to now only the 2.4-dichlorophenoxypropionic acid (I, $R = CH_3$) has been resolved into optical antipodes (1, 2). Owing to the interesting plant physiological effects which have been reported for a number of optically active acids with similar structures, (3–6) it seemed desirable to prepare some other optically active acids containing the 2.4-dichlorophenoxy and 2-methyl-4-chlorophenoxy structures.



I



II

α-(2-Methyl-4-chlorophenoxy)-propionic acid (II, $R = CH_3$) was resolved using (+)- and (−)-α-phenethylamine. The (+)-form of α-(2.4-dichlorophenoxy)-*n*-butyric acid (I, $R = C_2H_5$) was obtained over the strychnine salt and the (−)-form by means of (−)-phenethylamine.

The biological effects of these compounds are being studied by Dr. B. ÅBERG and will be described elsewhere by him. According to Dr. ÅBERG the (+)-forms show very strong auxin activity. The activity of the (−)-form of the propionic acid derivative is about 900 times weaker than that of the (+)-form. Also the biological effect of the (−)-form of α-(2.4-dichlorophenoxy)-*n*-butyric acid is much smaller than that of the (+)-form; in this case the difference is about 500 times.

The main question from a stereochemical point of view is, if the biologically more active forms have the D-configuration, in conformity with other α-aryloxyalkylcarboxylic acids (7). This problem was solved by means of the method of quasi-racemates (8). Preliminary experiments were carried out using the contact method (9). It was

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[1] Part VI: Arkiv Kemi *6*, 355 (1953).

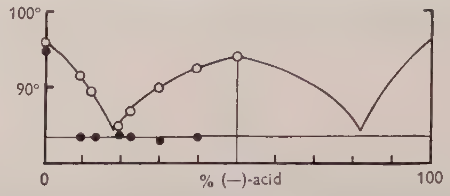

Fig. 1. (+)- and (−)-α-(2-methyl-4-chlorophenoxy)-propionic acid.

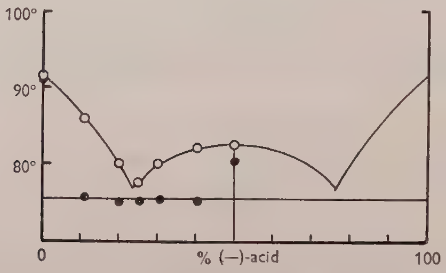

Fig. 2. (+)- and (−)-α-(2.4-dichlorophenoxy)-*n*-butyric acid.

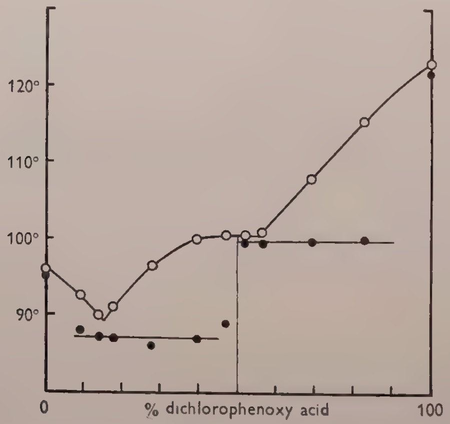

Fig. 3. (−)-α-(2-Methyl-4-chlorophenoxy)-propionic acid and (+)-α-(2.4-dichlorophenoxy)-propionic acid.

found that α-(2-methyl-4-chlorophenoxy)-propionic acid (II, R = CH_3) and α-(2.4-dichlorophenoxy)-propionic acid (I, R = CH_3), with opposite directions of optical rotation, formed a molecular compound. The acids with the same direction of rotation did not give such a compound but were mutually soluble in the solid state with a peritectic point at 98°. Thus, the molecular compound probably is quasi-racemic. In the same way it was found that α-(2.4-dichlorophenoxy)-propionic acid (I, R = CH_3) and the homologous *n*-butyric acid (I, R = C_2H_5) with opposite directions of optical rotation formed a molecular compound, probably quasi-racemic. In this case too it was found that the acids with the same direction of rotation were mutually soluble to a large extent.

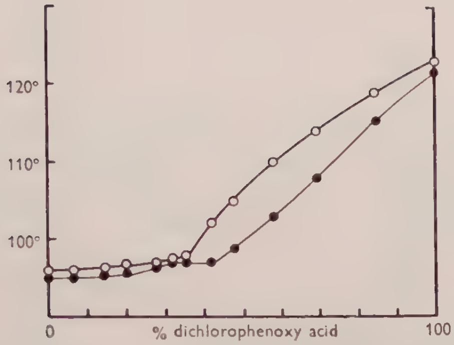

Fig. 4. (+)-α-(2-Methyl-4-chlorophenoxy)-propionic acid and (+)-α-(2.4-dichlorophenoxy)-propionic acid.

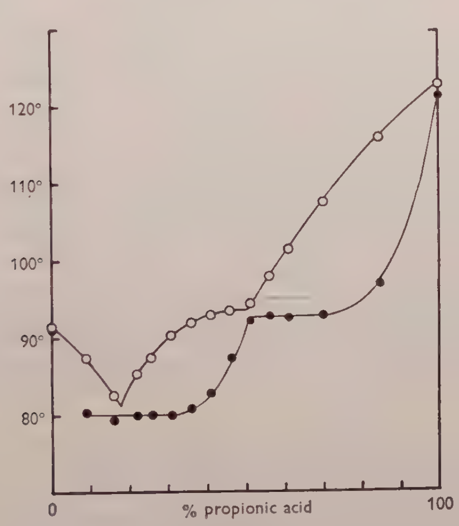

Fig. 5. (+)-α-(2.4-Dichlorophenoxy)-propionic acid and (−)-α-(2.4-dichlorophenoxy)-*n*-butyric acid.

To get more evidence for the quasi-racemic nature of the molecular compounds, melting point diagrams were studied. As seen from Figs. 1 and 2 the racemic acids are true racemates, but their tendency to form racemic compounds is small, as is shown by the fact, that the middle part of each diagram is not very pronounced. As the same applies to α-(2.4-dichlorophenoxy)-propionic acid it is not surprising that the parts of the melting point diagrams in Figs. 3 and 5 which represent the quasi-racemates are rather undeveloped. The melting point curves in Figs. 4 and 6 may be classified as a combination of the Roozeboom types IV and V. Evidently, the acids with the same direction of rotation are mutually soluble in the solid state to a large extent.

The results of the thermal analysis by the contact method agree very well with the corresponding melting point curves. The use of the contact method for explan-

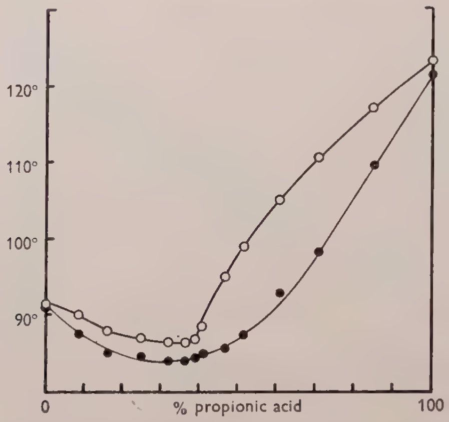

Fig. 6. (+)-α-(2.4-Dichlorophenoxy)-propionic acid and (+)-α-(2.4-dichlorophenoxy)-*n*-butyric acid.

atory investigations by the method of quasi-racemates is both reliable and rapid, as has been previously pointed out (10).

By definition the components of a quasi-racemic compound have opposite configurations. It has previously been proved that (+)-α-(2.4-dichlorophenoxy)-propionic acid belongs to the D-series (7) and accordingly the (+)-forms of the acids studied here have the D-configuration.

In Table 1 the optical activity data for (+)-α-(2.4-dichlorophenoxy)-propionic acid in different solvents have been arranged to give a decreasing series. It is seen that the data for the *n*-butyric acid then give a decreasing series too. This fact is added proof that the acids in question have the same configuration.

Table 1. $[\alpha]_D^{25}$ for (+)-α-(2.4-dichlorophenoxy)-propionic acid (I, $R = CH_3$) and (+)-α-(2.4-dichlorophenoxy)-*n*-butyric acid (I, $R = C_2H_5$) in different solvents.

Solvent	I, $R = CH_3$	I, $R = C_2H_5$
Acetone	+35.2°	+52.1°
Alcohol	+28.1°	+41.8°
Chloroform	+21.0°	+35.3°
Water (Na-salt)	−14.1°	+ 5.6°
Benzene	−17.1°	− 7.4°

Experimental

Racemic acids. These were prepared in the usual way by refluxing equimolecular quantities of ethyl α-bromopropionate and α-bromo-*n*-butyrate respectively and the appropriate sodium phenolate, followed by hydrolysis of the ester. The *(2-methyl-4-chlorophenoxy)-propionic acid* was recrystallised alternately from 80 % formic acid and petroleum (b.p. 60–80°). M.p. 93–94°.

86.85 mg acid: 8.210 ml 0.04955-*N* NaOH
$C_{10}H_{11}O_3Cl$ calc. equiv. wt. 214.6
found » » 213.4

The *2.4-dichlorophenoxy-n-butyric* acid was recrystallised several times from petroleum (b.p. 60–80°). It was found to be dimorphic. The product which crystallised from petroleum melted at 80–82°. When the melt was kept at 80–83° a new modification crystallised very slowly. It melted at 84.5°.

0.3239 g acid: 12.97 ml 0.1001-*N* NaOH
$C_{10}H_{10}O_3Cl_2$ calc. equiv. wt. 249.1
found » » 249.5

Preliminary experiments on resolution. α-*(2-Methyl-4-chlorophenoxy)-propionic acid* gave crystalline salts with strychnine, quinine and α-phenethylamine. Only the latter base gave a satisfactory resolution. The (+)-amine was used for the isolation of the (+)-acid and the (−)-amine for the (−)-acid.

α-*(2.4-Dichlorophenoxy)-n-butyric* acid yielded crystalline salts with strychnine, quinine, cinchonidine and α-phenethylamine. From the quinine and (−)-phenethylamine salts a lævorotatory acid was obtained and the latter base was used for the isolation of the (−)-acid. The other bases yielded dextrorotatory products; strychnine gave the best resolution and was used for the preparation of the (+)-acid.

(+)-α-(2-Methyl-4-chlorophenoxy)-propionic acid. Equimolecular quantities of the racemic acid (28.2 g, 0.13 mole) and (+)-α-phenethylamine (16.0 g) were dissolved in a hot mixture of ethanol (100 ml) and water (500 ml). The solution was set aside to crystallise in a refrigerator over night. A small sample was treated with hydrochloric acid and the organic acid was taken up in ether, which was evaporated. The rotatory power of this product was measured in abs. alcohol. The salt was recrystallised until the optical activity did not increase any more (Table 2).

Table 2. Recrystallisation of the (+)-α-phenethylamine salt of α-(2-methyl-4-chlorophenoxy)-propionic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	[α]D of the acid
	Ethanol	Water		
1	100	500	29.8	+ 3.5°
2	100	500	17.5	+12.5°
3	75	375	12.2	+17.3°
4	50	250	9.35	+18.7°
5	50	250	7.35	+19.5°
6	40	200	5.65	+19.2°

The organic acid was isolated from the last salt fraction and the crude acid (3.4 g) was recrystallised from 75% formic acid. 2.77 g (+)-acid were collected as small, white needles. M.p. 95–96°.

86.39 g (+)-acid: 8.210 ml 0.04888-*N* NaOH
$C_{10}H_{11}O_3Cl$ calc. equiv. wt. 214.6
found » » 215.3

0.1836 g dissolved in abs. *alcohol* to 20.06 ml: $2\,\alpha_D^{25} = +0.349°$. $[\alpha]_D^{25} = +19.0°$; $[M]_D^{25} = +40.9°$
0.1876 g » » *acetone* to 20.06 ml: $2\,\alpha_D^{25} = +0.548°$. $[\alpha]_D^{25} = +29.3°$; $[M]_D^{25} = +62.9°$
0.2197 g » » *chloroform* to 20.06 ml: $2\,\alpha_D^{25} = +0.357°$. $[\alpha]_D^{25} = +16.3°$; $[M]_D^{25} = +35.0°$
0.2002 g » » *benzene* to 20.06 ml: $2\,\alpha_D^{25} = +0.315°$. $[\alpha]_D^{25} = +15.8°$; $[M]_D^{25} = +33.9°$
0.1873 g neutralised with *aqueous NaOH* and made up to 20.06 ml: $2\,\alpha_D^{25} = +0.099°$. $[\alpha]_D^{25} = +5.3°$; $[M]_D^{25} = +11.4°$.

(−)-α-(2-Methyl-4-chlorophenoxy)-propionic acid. The organic acid was recovered from the mother liquours 1 and 2 (Table 2). This acid (16.5 g, 0.077 mole, $[\alpha]_D = -8°$ in alcohol) and an equimolecular quantity of (−)-α-phenethylamine (9.3 g) were dissolved in a hot mixture of alcohol (100 ml) and water (500 ml). The solution was set aside in a cool place and the crystalline product was collected next day. The rotatory power of a small sample of the acid was measured (in alcohol) in the usual way. The course of the purification is seen from Table 3.

Table 3. Recrystallisation of the (−)-α-phenethylamine salt of α-(2-methyl-4-chlorophenoxy)-propionic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Ethanol	Water		
1	100	500	14.4	−16.8°
2	75	375	10.6	−18.0°
3	60	300	7.8	−17.8°
4	50	250	5.17	−17.9°

From the salt fraction 4, 3.23 g of the crude (−)-acid were isolated. It was recrystallised from 75% formic acid, yielding 2.35 g of the pure (−)-acid. M.p. 94.5–96°.

83.55 mg (−)-acid: 8.010 ml 0.04888-*N* NaOH
$C_{10}H_{11}O_3Cl$ calc. equiv. wt. 214.6
found » » 213.4

0.1740 g dissolved in abs. *alcohol* to 20.06 ml: $2\,\alpha_D^{25} = -0.326°$. $[\alpha]_D^{25} = -18.8°$; $[M]_D^{25} = -40.3°$.

(+)-α-(2.4-Dichlorophenoxy)-*n*-butyric acid. Racemic acid (15.0 g 0.06 mole) and strychnine (20.1 g, 0.06 mole) were dissolved in hot alcohol (300 ml). Water (400 ml) was added and the solution was allowed to crystallise in a cool place over night. The salt was recrystallised according to Table 4, where $[\alpha]_D$ refers to the rotatory power of the acid in alcohol.

Table 4. Recrystallisation of the strychnine salt of α-(2.4-dichlorophenoxy)-*n*-butyric acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Ethanol	Water		
1	300	400	31.2	—
2	300	400	12.8	—
3	150	200	10.6	+40.2°
4	125	175	8.75	+41.6°
5	125	175	8.25	+41.4°

The last salt fraction was treated with hydrochloric acid and the organic acid taken up in ether, which was driven off. The crude acid (3.5 g) was recrystallised from petroleum (b.p. 60–80°). The pure (+)-acid (3.35 g) melted at 91–91.6°.

95.97 mg (+)-acid: 7.855 ml 0.04888-*N* NaOH
$C_{10}H_{10}O_3Cl_2$ calc. equiv. wt. 249.1
found » » 249.9

0.2052 g dissolved in abs. *alcohol* to 10.00 ml: $\alpha_D^{25} = +0.858°$. $[\alpha]_D^{25} = +41.8°$; $[M]_D^{25} = +104.2°$.
0.2279 g » » *acetone* to 20.06 ml: $2\alpha_D^{25} = +1.183°$. $[\alpha]_D^{25} = +52.1°$; $[M]_D^{25} = +129.7°$.
0.1846 g » » *chloroform* to 20.06 ml: $2\alpha_D^{25} = +0.650°$. $[\alpha]_D^{25} = +35.3°$; $[M]_D^{25} = +88.0°$.
0.2428 g » » *benzene* to 20.06 ml: $2\alpha_D^{25} = -0.179°$. $[\alpha]_D^{25} = -7.4°$; $[M]_D^{25} = -18.4°$.
0.2116 g neutralised with *aqueous NaOH* and made up to 20.06 ml: $2\alpha_D^{25} = +0.118°$. $[\alpha]_D^{25} = +5.6°$; $[M]_D^{25} = +13.9°$.

(−)-α-(2.4-Dichlorophenoxy)-*n*-butyric acid. From the mother liquor 1 above, a lævorotatory acid ($[\alpha]_D = -19°$ in alcohol) was recovered. This acid (7.2 g, 0.029 mole) and (−)-α-phenethylamine (3.5 g, 0.029 mole) were dissolved in hot, 30% alcohol. The solution was set aside in a refrigerator and the crystals were collected next day. The purification of the salt was carried out as seen from Table 5. The rotatory power of the acid isolated from small samples of the salt fractions was measured in alcohol as usual.

Table 5. Recrystallisation of the (−)-α-phenethylamine salt of α-(2.4-dichlorophenoxy)-*n*-butyric acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Ethanol	Water		
1	45	110	6.2	−29.9°
2	25	65	5.0	−35.9°
3	25	65	4.3	−36.1°
4	35	50	2.70	−36.2°

The salt fraction 4 was decomposed and the acid liberated in the usual way. The crude acid (2.0 g) was recrystallised from petroleum (b.p. 60–80°). 1.1 g of the (−)-acid was collected as very small, white needles, m.p. 90.5–91.5°.

105.85 mg (−)-acid: 8.660 ml 0.04888-*N* NaOH
$C_{10}H_{10}O_3Cl_2$ calc. equiv. wt. 249.1
found » » 250.1

0.1354 g dissolved in abs. *alcohol* to 10.02 ml: $\alpha_D^{25} = -0.559°$. $[\alpha]_D^{25} = -41.4°$; $[M]_D^{25} = -103.1°$.

Melting point diagrams. The samples were prepared by dissolving weighed quantities of the components in acetone. After evaporation of the solvent, the residue was powdered and dried. All melting points were determined with a Kofler hot stage microscope (11). The results are found in Tables 6–11 and Figs. 1–6, where the composition is given in mole-%. A hot stage microscope was used for the study of contact preparations too.

Table 6. (+)- and (−)-II($R=CH_3$).

% (−)-form	M.p.
0.0	95 –96°
9.2	83.5–91.5°
13.2	83.5–89.5°
19.2	83.5–85°
22.4	83.5–87°
29.9	83 –90°
39.6	83.5–92.5°
50.0	93 –94°

Table 7. (+)- and (−)-I($R=C_2H_5$).

% (−)-form	M.p.
0.0	91 –91.5°
11.1	75.5–86°
19.9	75 –80°
25.2	75 –77.5°
30.4	75.5–80°
40.6	75 –82°
50.0	80 –82°

Table 8. (−)-II($R=CH_3$) and (+)-I($R=CH_3$).

% (+)-I	M.p.
0.0	95 – 96°
9.2	88 – 92.5°
13.9	87 – 90°
17.8	87 – 91°
27.8	86 – 96.5°
39.5	87 –100°
47.2	89 –100.5°
52.0	99.5–100.5°
56.5	99.5–101°
69.2	100 –108°
82.9	100 –115.5°
100.0	121.5–123°

Table 9. (+)-II($R=CH_3$) and (+)-I($R=CH_3$).

% (+)-I	M.p.
0.0	95 – 96°
6.7	95 – 96°
14.4	95.5– 96.5°
20.6	95.5– 97°
27.8	96.5– 97°
32.1	97 – 97.5°
35.4	97 – 98°
42.0	97 –102°
47.8	99 –105°
58.2	103 –110°
69.2	108 –114°
84.5	115.5–119°
100.0	121.5–123°

Table 10. (+)-I($R=CH_3$) and (−)-I($R=C_2H_5$).

% (+)-I($R=CH_3$)	M.p.
0.0	91 – 91.5°
8.9	80.5– 87.5°
16.1	79.5– 82.5°
21.9	80 – 85.5°
25.6	80 – 87.5°
30.7	80 – 90.5°
35.8	81 – 92°
40.8	83 – 93°
46.1	87.5– 93.5°
51.2	92 – 94.5°
56.2	93 – 98°
61.0	92.5–101.5°
70.1	93 –107.5°
84.5	97 –116.5°
100.0	121.5–123°

Table 11. (+)-I($R=CH_3$) and (+)-I($R=C_2H_5$).

% (+)-I($R=CH_3$)	M.p.
0.0	91 – 91.5°
9.0	87.5– 90°
16.2	85 – 87°
24.9	84.5– 87°
31.9	84 – 86.5°
36.4	84 – 86.5°
41.1	85 – 88.5°
47.1	85.5– 95°
51.8	87.5– 99.5°
61.2	93 –105°
71.1	98 –110.5°
84.8	109.5–117°
100.0	121.5–123°

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SUMMARY

α-(2-Methyl-4-chlorophenoxy)-propionic acid and α-(2.4-dichlorophenoxy)-*n*-butyric acid have been resolved into optical antipodes. The rotatory power has been measured in different solvents. The optically active acids have been sterically connected to the glyceric aldehyde system. The (+)-forms, which have much greater auxin activity than the corresponding (−)-forms, have the D-configuration. The acids investigated here constitute further examples of the stereochemical specificity, which seems to be a general property of plant growth regulators of the α-aryloxyalkylcarboxylic acid type.

Department of Organic Chemistry, Royal Agricultural College, Uppsala, June 1953.

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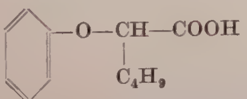
Stereochemical studies on plant growth regulators. VIII[1]

The resolution of α-phenoxy- and α-(2-naphthoxy)-*n*-caproic acid into optical antipodes

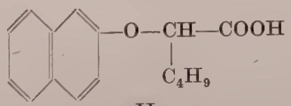
By MAGNUS MATELL

Previous papers of this series have dealt with the preparation of some optically active derivatives of propionic and *n*-butyric acid and the establishment of their steric relations (1–7). In all the cases, where the configurations could be established, it was found, that those optically active forms, which have greater auxin activity than their corresponding antipodes, belong to the D-series. It seems reasonable to suppose, that this stereochemical specificity is shown by the higher homologues of the α-aryloxyalkylcarboxylic acids too. An extension of these studies to the *n*-caproic acids therefore may seem to be a secondary task. However, Dr. B. ÅBERG, who is studying the biological effects of compounds of this type, has undertaken some preliminary experiments on the racemic forms of higher homologues in the α-aryloxyalkylcarboxylic acid series. The results were such, that a more thorough investigation of optically active *n*-caproic acids seemed justified (private communication).

The racemic α-phenoxy-*n*-caproic acid (I) and α-(2-naphthoxy)-*n*-caproic acid (II), which seem not to have been described in the literature before, were prepared in the usual manner from ethyl α-bromo-*n*-caproate and the appropriate sodium phenolate.



I



II

The preliminary experiments on resolution did not give encouraging results when the common bases were used. Of the 16 different salts, which were prepared, only 7 crystallised and with two exceptions the rotatory power of the acids, obtained from these salts, was very small. However, it has recently been pointed out that 2-amino-1-phenylpropane (β-phenyl*iso*propylamine) may be valuable as a complement to the common bases (8). Both the acids I and II gave crystalline products with this base and the resolving power was satisfactory. The resolutions were then carried out without any difficulties using β-phenyl*iso*propylamine.

The enantiomorphic acids have not yet been sterically connected to the previously described α-aryloxyalkylcarboxylic acids. However, optical activity measurements in different solvents indicate that (+)-α-phenoxy-*n*-caproic acid has the D-configuration. As seen from Table 1 the activity data for (−)-α-phenoxy-*n*-butyric acid have been arranged to give an increasing series. The corresponding data for (+)-α-phenoxy-*n*-caproic acid then give a decreasing series. Thus it is probable that these two compounds have opposite configurations. As (−)-α-phenoxy-*n*-butyric acid has been shown to belong to the L-series (6) the (+)-α-phenoxy-*n*-caproic acid probably has the D-configuration. A similar connection was not found for the corresponding 2-naphthoxy acids.

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[1] Part VII: Arkiv Kemi, 6, 365 (1953).

Table 1. $[\alpha]_D^{25}$ for (−)-α-phenoxy-*n*-butyric acid [(−)-POB] and (+)-α-phenoxy-*n*-caproic acid [(+)-POC].

Solvent	(−)-POB	(+)-POC
Acetone	−65.5°	+48.0°
Alcohol	−51.4°	+37.6°
Chloroform	−36.4°	+24.5°
Water (Na-salt)	−20.6°	+16.2°
Benzene	− 5.2°	+ 2.8°

Experimental

rac.-α-Phenoxy-n-caproic acid. Sodium was dissolved in abs. alcohol and equimolecular quantities of phenol and α-bromo-*n*-caproic acid were added. The solution was refluxed for two hours, and excess of dilute sodium hydroxide was added. Boiling was continued for another hour, the alcohol was distilled off and the residue acidified with hydrochloric acid. An oil separated and after one night it crystallised. As the product was found to contain unreacted phenol, it was treated with ether and dilute sodium bicarbonate. When the latter was acidified an oil separated which however crystallised in a few minutes. The yield of the crude acid was 81%. It was recrystallised three times from 80% formic acid. M.p. 73–74°.

84.64 g acid: 8.385 ml 0.04839-*N* NaOH
$C_{12}H_{16}O_3$ calc. equiv. wt. 208.3
found » » 208.6

Table 2. Recrystallisation of the (−)-β-phenyl*iso*propylamine salt of α-phenoxy-*n*-caproic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Alcohol	Water		
1	85	195	14.5	+ 6.0°
2	75	175	11.2	+14.8°
3	60	140	8.3	+26.3°
4	50	110	6.5	+33.7°
5	40	90	5.8	+36.0°
6	35	80	4.3	+36.8°
7	30	70	3.6	+37.3°
8	25	60	3.11	+37.3°

(+)-*α-Phenoxy-n-caproic acid.* Racemic acid (11.5 g, 0.055 mole) and (−)-β-phenyl*iso*propylamine (7.4 g, 0.055 mole) were dissolved in a hot mixture of 85 ml alcohol and 195 ml water. The salt was allowed to crystallise in a cool place for some hours. The optical activity of the acid, liberated from a small sample, was measured in alcohol. The salt was recrystallised several times according to Table 2.

From the last salt fraction 1.78 g of the crude (+)-acid were obtained. Recrystallisation from 50% formic acid yielded 1.42 g of the pure (+)-form, m.p. 75–76.7°.

77.69 mg acid: 7.510 ml 0.04955-*N* NaOH
$C_{12}H_{16}O_3$ calc. equiv. wt. 208.3
found » » 208.8

0.1893 g dissolved in abs. *alcohol* to 20.06 ml: $2\alpha_D^{25} = +0.709°$. $[\alpha]_D^{25} = +37.6°$; $[M]_D^{25} = +78.2°$.
0.1834 g » » *acetone* to 20.06 ml: $2\alpha_D^{25} = +0.877°$. $[\alpha]_D^{25} = +48.0°$; $[M]_D^{25} = +99.9°$.
0.1710 g » » *chloroform* to 20.06 ml: $2\alpha_D^{25} = +0.418°$. $[\alpha]_D^{25} = +24.5°$; $[M]_D^{25} = +51.1°$.
0.1835 g » » *benzene* to 20.06 ml: $2\alpha_D^{25} = +0.051°$. $[\alpha]_D^{25} = +\ 2.8°$; $[M]_D^{25} = +\ 5.8°$.
0.1638 g neutralised with *aqueous NaOH* and made up to 20.06 ml: $\alpha_D^{25} = +0.132°$. $[\alpha]_D^{25} = +16.2°$; $[M]_D^{25} = +33.7°$.

(−)-*α-Phenoxy-n-caproic acid.* From the mother liquors 1–3 (Table 2) a lævorotating acid ($[\alpha]_D = -19°$ in alcohol) was regenerated. This acid (6.15 g, 0.03 mole) and an equivalent quantity of (+)-β-phenyl*iso*propylamine (4.0 g) were dissolved in 70 ml of alcohol and 160 ml of water in the hot. The salt, which separated on cooling, was recrystallised as seen from Table 3. The rotatory power of the acid was measured in alcohol as usual.

Table 3. Recrystallisation of the (+)-β-phenyl*iso*propylamine salt of α-phenoxy-*n*-caproic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Alcohol	Water		
1	75	160	7.3	−29.9°
2	60	130	6.1	−34.8°
3	50	105	5.1	−36.3°
4	40	90	4.2	−36.9°
5	30	70	3.52	−36.9°

Salt fraction 5 was decomposed and the organic acid isolated. The crude acid (2.0 g) was recrystallised from 50% formic acid. The pure (−)-acid melted at 75–76.4°.

73.54 mg acid: 7.095 ml 0.04955-*N* NaOH
$C_{12}H_{16}O_3$ calc. equiv. wt. 208.3
found » » 209.2

0.1731 g dissolved in abs. *alcohol* to 20.06 ml: $2\alpha_D^{25} = -0.648°$. $[\alpha]_D^{25} = -37.5°$; $[M]_D^{25} = -78.2°$.

rac.-α-(2-Naphthoxy)-n-caproic acid. This was prepared in 82% yield according to the same method as the phenoxy acid. It was recrystallised from formic acid and melted at 108–110°.

99.27 mg acid: 7.865 ml 0.04888-*N* NaOH
$C_{16}H_{18}O_3$ calc. equiv. wt. 258.3
found » » 258.2

(+)-α-(2-Naphthoxy)-n-caproic acid. Racemic acid (15.5 g, 0.06 mole) and β-phenyl*iso*propylamine (8.1 g, 0.06 mole) were dissolved in a hot mixture of alcohol (300 ml) and water (120 ml). The solution was set aside in a refrigerator over night. The crystalline salt was collected and the organic acid was liberated from a small sample. The rotatory power of this acid was measured in abs. alcohol. The salt was recrystallised according to Table 4.

Table 4. Recrystallisation of the (−)-β-phenyl*iso*propylamine salt of α-(2-naphthoxy)-*n*-caproic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Alcohol	Water		
1	300	120	11.9	+39.7°
2	150	60	9.6	+56.4°
3	100	40	8.5	+58.2°
4	85	45	7.7	+57.5°
5	80	40	7.1	+58.8°

From the last salt fraction 4.5 g of the crude (+)-acid were obtained. It was recrystallised from petroleum (b.p. 60–80°). The pure acid melted at 134.5–135.2°.

100.10 mg acid: 7.800 ml 0.04955-*N* NaOH
$C_{16}H_{18}O_3$ calc. equiv. wt. 258.3
found » » 259.0

0.1885 g dissolved in abs. *alcohol* to 20.06 ml: $2\alpha_D^{25} = +1.102°$. $[\alpha]_D^{25} = +58.6°$; $[M]_D^{25} = +151.5°$.
0.1818 g » » *acetone* to 20.06 ml: $2\alpha_D^{25} = +1.324°$. $[\alpha]_D^{25} = +73.0°$; $[M]_D^{25} = +188.7°$.
0.1968 g » » *chloroform* to 20.06 ml: $2\alpha_D^{25} = +0.736°$. $[\alpha]_D^{25} = +37.5°$; $[M]_D^{25} = +\ 96.9°$.
0.1943 g » » *benzene* to 20.06 ml: $2\alpha_D^{25} = +1.197°$. $[\alpha]_D^{25} = +61.8°$; $[M]_D^{25} = +159.6°$.
0.1669 g neutralised with *aqueous NaOH* and made up to 20.06 ml: $2\alpha_D^{25} = +0.661°$. $[\alpha]_D^{25} = +39.7°$; $[M]_D^{25} = +102.6°$.

(−)-α-(2-Naphthoxy)-n-caproic acid. The organic acid was regenerated from mother liquor 1 (Table 4). This acid (7.75 g, 0.03 mole, $[\alpha]_D = -35°$ in alcohol) and an equimolecular quantity of β-phenyl*iso*propylamine (4.05 g) were dissolved in a hot mixture of alcohol (150 ml) and water (60 ml). The salt, which separated on cooling, was recrystallised according to Table 5: $[\alpha]_D$ refers to the optical activity of the acid measured in alcohol.

Table 5. Recrystallisation of the (+)-β-phenyl*iso*propylamine salt of α-(2-naphthoxy)-*n*-caproic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Alcohol	Water		
1	150	60	8.8	−51.1°
2	120	50	6.9	−56.3°
3	95	40	5.9	−57.4°
4	80	35	5.15	−57.0°

The crude acid, which was isolated from the last salt fraction, was recrystallised from petroleum (b.p. 60–80°). 3.12 g of the pure (−)-acid were collected. M.p. 134.5–135.2°.

104.86 mg acid: 8.185 ml 0.04955-*N* NaOH
$C_{16}H_{18}O_3$ calc. equiv. wt. 258.3
found » » 258.5

0.1948 g dissolved in abs. *alcohol* to 20.06 ml: $2\alpha_D^{25} = -1.136°$. $[\alpha]_D^{25} = -58.5°$; $[M]_D^{25} = -151.1°$

The author is indebted to Prof. N. HELLSTRÖM, the head of this department, for his kind interest and for all facilities put at the author's disposal. Thanks are due to Mr A. AAMISEPP for technical assistance in the preparation of the racemic acids. Part of the expenses involved have been defrayed by a grant from *Statens Naturvetenskapliga Forskningsråd*, which is gratefully acknowledged.

SUMMARY

α-Phenoxy-*n*-caproic acid (I) and α-(2-naphthoxy)-*n*-caproic acid (II) have been synthesised and resolved into optical antipodes. The resolution was carried out by means of β-phenyl*iso*propylamine. The rotatory power has been measured in different solvents.

Department of Organic Chemistry, Royal Agricultural College, Uppsala, June 1953.

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5. M. MATELL, Ibid. *6*, 251 (1953).
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Tryckt den 11 september 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 37

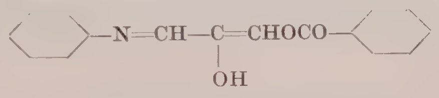
Mitgeteilt am 3. Juni 1953 durch HANS VON EULER und KARL MYRBÄCK

Zur Kenntnis der Reduktonanile

Von HANS HASSELQUIST

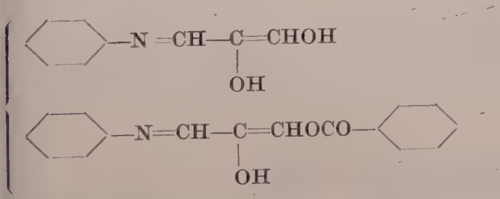
Bei der Umsetzung des Redukton-mono-anil-Na-Salzes mit Benzoylchlorid in Äther erhielten COCKER, O'MEARA, SCHWARZ und STUART (1) ein Mono-benzoyl-redukton-mono-anil von der Zusammensetzung

I.

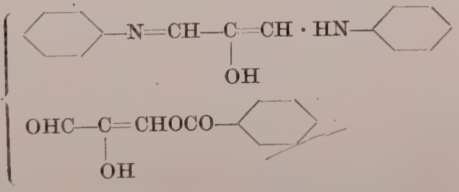


Man kann noch nicht entscheiden, ob die Stellung des Benzoylrestes so ist, wie COCKER angegeben hat, oder ob der Benzoylrest am Kohlenstoffatom C_2 sitzt. Die Aufgabe, durch die folgenden Versuche etwas Licht in diese Frage zu bringen, stiess auf erhebliche Schwierigkeiten, weil der Benzoylrest sehr lose angelagert ist, wie im Folgenden gezeigt wird. Dasselbe Anil (I), welches oben formuliert ist, wurde als Hauptprodukt erhalten, wenn das von EULER und HASSELQUIST (2) isolierte Mono-benzoyl-redukton mit Anilin in Benzollösung umgesetzt wurde. Dabei wurde auch in kleinen Mengen eine Substanz erhalten, welche in orangeroten Prismen gut krystallisierte. Die letztgenannte Substanz konnte auch als Hauptprodukt erhalten werden, wenn die Kondensation in Methanollösung bei + 10° ausgeführt wurde. Sowohl Analyse als auch titrimetrische Bestimmung mit Tillmans Reagens (im Folgenden als TR abgekürzt) ohne Zusatz von Alkali deuten darauf hin, dass die Bildung einer Komplexverbindung folgender Konstitution vorliegt:

II

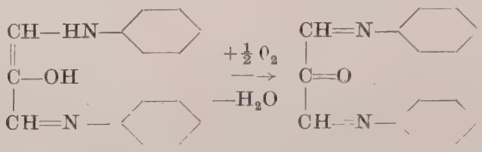


III



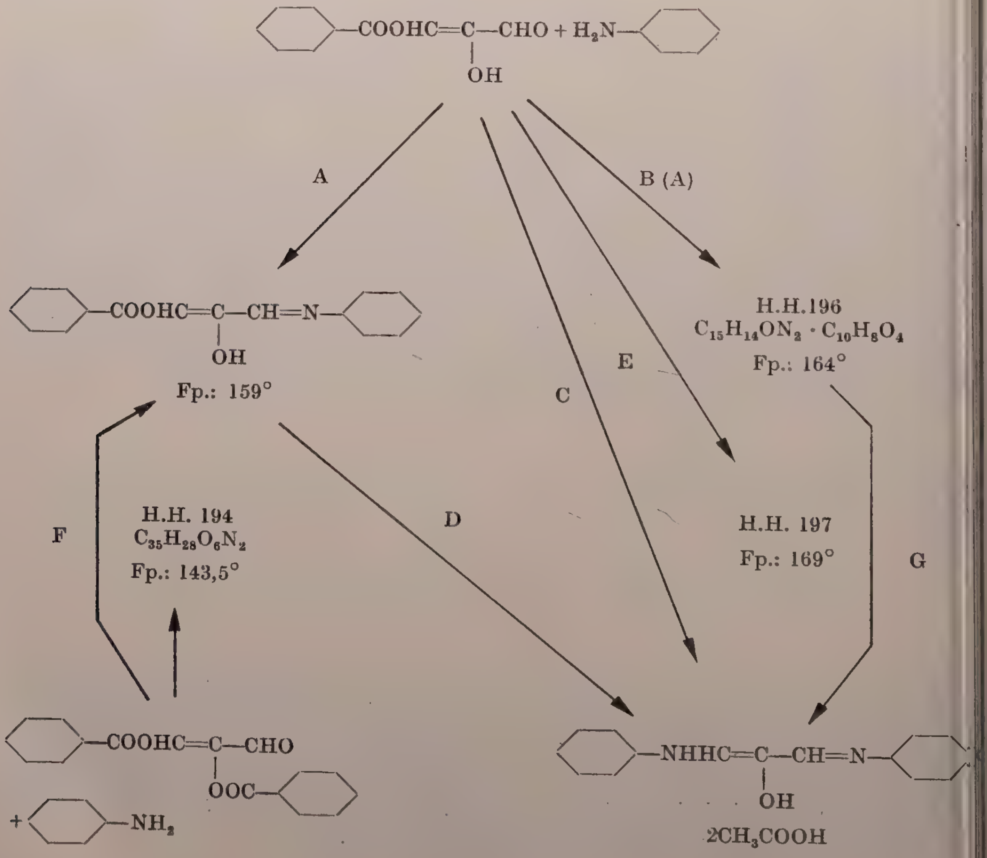
Formel III ist die wahrscheinlichste, da das in dieselbe eingehende Reduktonanil (3) sowie dessen Salze stark gefärbt sind. In Formel II sind dagegen die beiden Komponenten, jede für sich, farblos. Für eine Komplexverbindung sprechen auch die Umstände, dass die Substanz durch Überhitzen bei der Umkrystallisation zerstört wird, ferner dass sie bei Zusatz von Essigsäure bei gewöhnlicher Temperatur leicht in ein essigsaures Salz des Reduktondianils übergeht.

Dass die Substanz TR ohne Zusatz von Alkali entfärbt beruht darauf, dass sowoh das Reduktonanil als dessen essigsaures Salz ohne Alkali-Zusatz TR verbrauchen (siehe experimenteller Teil). Die Oxydation verläuft also in folgender Weise:


CH—HN—
C—OH
CH=N —
$+\frac{1}{2}O_2$
$-H_2O$
CH=N—
C=O
CH—N—

zu Mesoxaldialdehyd-dianil.

Führt man die Kondensation des Mono-benzoyl-reduktons mit Anilin in Benzollösung in Gegenwart von Essigsäure aus, so erhält man nicht die erwähnte Komplexverbindung sondern nur das von COCKER (1) isolierte essigsaure Salz des Redukton-dianils. Daraus folgt, dass der Benzoylrest leicht abgespalten wird. So tritt diese Spaltung auch ein, wenn man das Mono-benzoyl-redukton-mono-anil mit Anilin in Benzol in Gegenwart von Essigsäure umsetzt, um ev. ein Mono-benzoyl-redukton-dianil zu erhalten; indessen wird dabei nur das oben beschriebene essigsaure Salz ohne Benzoylrest erhalten.


—COOHC=C—CHO + H_2N—
OH
A
B (A)
—COOHC=C—CH=N—
OH
Fp.: 159°
H.H.196
$C_{15}H_{14}ON_2 \cdot C_{10}H_8O_4$
Fp.: 164°
E
C
D
F
H.H. 194
$C_{35}H_{28}O_6N_2$
Fp.: 143,5°
H.H. 197
Fp.: 169°
G
—COOHC=C—CHO
OOC—
+ —NH_2
—NHHC=C—CH=N—
OH
$2CH_3COOH$

Bei Wiederholung des COCKER'schen Versuches (1) unter Kondensation des Dibenzoyl-reduktons mit Anilin in Benzol in der Wärme erhielt ich als Endprodukt Mono-benzoyl-redukton-mono-anil. Dadurch erhält man eine gewisse Erklärung für den von COCKER für das berechnete Dibenzoyl-redukton-anil bei der Analyse erhaltenen hohen N-Wert. Beim Kochen des Mono-benzoyl-reduktons in Methanol tritt eine Veränderung des Ausgangsmateriales ein. Nach dem Einengen der Methanollösung konnte das Ausgangsmaterial nicht mehr zurückgewonnen werden, sondern als Rest wurde ein farbloses Öl erhalten, das bei Umsetzung mit Anilin in etwas Methanol bei 10° in geringer Menge als farblose Stäbchen krystallisieren der Substanz erschien. Dieselbe schmolz bei 169–170° unter Zersetzung und Dunkelfärbung und war nicht identisch mit Mono-benzoyl-redukton-mono-anil, das bei 159–160° ohne Zersetzung und ohne Verfärbung schmilzt.

Hydrierungsversuche mit Mono-benzoyl-redukton wurden ausgeführt um ev. das entsprechende Benzoyl-glycerin zu erhalten, wodurch die Stellung des Benzoylrestes im Redukton festgestellt werden kann. Auch hier zeigte es sich, dass der Benzoylrest abgespalten wird und zwar unter Bildung von Benzoesäure, welche isoliert werden konnte. Der Rückstand bestand aus einem farblosen in Wasser schwerlöslichen Öl das mit Glycerin nicht identisch war.

A) Umsetzung von Mono-benzoyl-redukton mit Anilin in Benzol

500 mg Mono-benzoyl-redukton wurden in der Wärme in möglichst wenig Benzol gelöst, worauf 0,25 ml Anilin ohne Wärmezufuhr zugesetzt wurden. Dabei färbte sich die Lösung gelb, gelbrot bis braun wobei eine kleine Menge orangegefärbter Prismen ausfielen, die abfiltriert und mit wenig Benzol gewaschen wurden. Ausbeute 30 mg Substanz vom Schmpt 156–157°. Nach Umkristallisieren aus Aceton stieg der Schmpt auf 164–165° (unter Zersetzung).

Die Substanz 196 ist ziemlich schwerlöslich in allen übrigen organischen Lösungsmitteln. Sie entfärbt TR *ohne* Zusatz von Alkali.

Die Sbst. 196 konnte auch in guter Ausbeute erhalten werden wenn man die Umsetzung in Methanol statt in Benzol ausführte. (Siehe unter B.)

Aus der Mutterlauge dieses Produktes konnte eine weitere Substanz durch ausfällen mit Petroleumäther isoliert werden. 200 mg einer gelbrot gefärbten Substanz wurden erhalten die nach fünfmaligem Umkristallisieren aus wenig Benzol den konstanten Schmpt 159–160 zeigten. Die so erhaltenen farblosen Kristalle erwiesen sich identisch mit dem von COCKER dargestellten Mono-Benzoyl-redukton-monoanil. Sie zeigte keine Schmpt-erniedrigung damit.

Die Substanz verbrauchte TR erst nach Zusatz von Alkali.

B) Umsetzung von Mono-benzoyl-redukton mit Anilin in kaltem Methanol

300 mg Mono-Benzoyl-redukton wurden mit 1 ml Methanol versetzt worauf (ohne Rücksicht auf die Lösung der Substanz) unter Abkühlung 0.15 ml Anilin zugegeben wurden. Das ganze blieb bei 10° stehen wobei die Substanz unter starker Gelbfärbung in Lösung ging. Das Reaktionprodukt kristallisierte in Form kleiner gelbroter Prismen aus. Ausbeute: 130 mg Sbst.

Die Sbst. 196 zeigte sich identisch mit dem zuerst unter A.) isoliertem.

Analyse:

Ber. für $C_{25}H_{22}O_5N_2$ (M = 430)	C 69,76 %	H 5,15 %	N 6,59 %
Gef.	69,63 %	5,22 %	6,52
	69,50 %	5,34 %	

Titration mit TR. 5,4 mg Sbst. gelöst in 10 ml Methanol werden titriert mit 0,0105-n TR, wobei ein Verbrauch von 2,45 ml eintrat.

$$E_{\text{ber}} = 215$$

$$E_{\text{gef}} = \frac{5,4}{0,0105 \cdot 2,45} = 210.$$

C) Umsetzung von Mono-benzoyl-redukton mit Anilin in Gegenwart von Essigsäure in Benzol

500 mg Mono-benzoyl-redukton wurden in der Wärme in möglichst wenig Benzol gelöst und mit 0,5 ml Eisessig sowie 0,5 ml Anilin versetzt und bei Zimmertemperatur stehen gelassen. Nach einer Stunde wurde das Ganze vorsichtig mit Petroleumäther unter Umrühren mit einem Glasstab versetzt, wobei das Reaktionprodukt in Form langer gelbroter Nadeln auskristallisierte.

Rohprodukt 200 mg vom Schmpt 156–157°. Umkristallisiert aus Benzol zeigte die Substanz den gleichen Schmelzpunkt. Sie war identisch mit dem von COCKER isoliertem essigsaurem Salz des Reduktonanils. COCKER gibt den Schmpt 150–153° an. Schmpt-erniedrigung bei der Mischung beider Substanzen trat nicht ein.

Die Substanz verbraucht TR *ohne* zusatz von Alkali.

Titration mit TR 8,1 mg Substanz in 10 ml Methanol gelöst wurden mit 0,0105-n TR titriert wobei ein Verbrauch von 4,5 ml eintrat.

$$E_{\text{ber}} = 179 \text{ für } C_{15}H_{14}ON_2 \cdot 2CH_3\,COOH$$

$$E_{\text{gef}} = \frac{8,1}{0,0105 \cdot 4,5} = 173.$$

D) Umsetzung von Mono-benzoyl-redukton-monoanil mit Anilin

500 mg Benzoyl-redukton-anil wurden in 2 ml Benzol gelöst und mit 0,5 ml Anilin sowie 0,5 ml Eisessig gelöst (ohne dass eine Reaktion eintrat) und auf dem Wasserbad zehn Minuten gekocht wobei die Anfangs farblose Lösung sich klar gelb färbte. Hierauf wurde vorsichtig Petroleumäther zugesetzt wobei lange gelbe Nadeln auskristallisierten. Ausbeute: 230 mg. Die Substanz erwies sich identisch mit dem unter C.) erhaltenen Dianilazetat.

E) Umsetzung des in Methanol umgewandelten Mono-benzoyl-reduktons mit Anilin

500 mg Benzoyl-redukton, in 2 ml Methanol gelöst, wurden 60 min. auf dem Wasserbad erhitzt (ein Versuch das Umlagerungsprodukt zu isolieren führte hier nur zu einem farblosen Öl das nicht zur Kristallisation gebracht werden kann) wonach unter Abkühlung bis 10° 0,25 ml Anilin zugesetzt wurden. Das ganze stand 20 Minuten wonach alles im Vacuum bei 20° eingedunstet wurde. Ein gelbbraunes Öl blieb zurück das mit etwas Benzol versetzt wurde. Beim Reiben mit einem Glasstab

kristallisierte ein Reaktionprodukt langsam in Form kleiner citronengelber Stäbchen aus, die mit etwas Benzol gewaschen wurden.

Nach mehrmaligem Eindunsten der Mutterlauge im Vacuum konnten noch weitere Substanzmengen isoliert werden; das gesamte Rohprodukt betrug 120 mg Substanz vom Schmpt 154–160° (unter Zersetzung). Die ganze Masse wurde in der Wärme in möglichst wenig Benzol gelöst. Es kristallisierte zuerst eine Substanz aus die mit Mono-benzoyl-redukton-monoanil identisch war, karakterisiert durch ihre spolförmige Kristallform. Sobald auch das zweite Produkt in Form kleiner fast farbloser Stäbchen auszukristallisiere begann wurde die Masse schnell abfiltriert und aus der Mutterlauge kristallisierten nun ausschliesslich die farblosen Stäbchen aus, die nach weiterem dreimaligem Umkristallisieren aus Benzol mit dem Konstanten Schmpt 169–170° (unter Zersetzung und Braunfärbung) erhalten wurden. Ausbeute: 15 mg.

Die Substanz 197 ist etwas leichter löslich in Benzol als das Mono-benzoyl-rekuktonanil, leicht löslich in Methanol, Azeton, Chloroform, schwerlöslich in Petrole- umäther.

Analyse:

Gef. C: 74,79 % H: 5,80 % N: 7,59 %

Wie sich durch spätere Versuche gezeigt hat erhält man bei der Umsetzung des Mono-benzoyl-reduktons mit Mono-methyl-anilin eine Substanz, die identisch ist mit derjenigen, die Cocker durch Umsetzung von Redukton-mono-methylanilin-Na-Salz mit Benzoylchlorid dargestellt hat. Dies spricht dafür, dass der Benzoylrest im Monobenzoyl-redukton am Kohlenstoffatom C_2 sitzt.

F) Umsetzung von Dibenzoyl-redukton mit Anilin nach der Vorschrift von Cocker

Dibenzoyl-redukton wurde noch Cocker mit Anilin umgesetzt. Die so erhaltenen abfiltrierten Kristalle zeigten einen Schmpt von 128–135°. Cocker gibt 126–128° an, nach Umkristallisierung aus Benzol. Die zuerst isolierte Substanz vom Schmpt 128–135° wurde nach sechsmaligem Umkristallisieren aus Bensol mit dem Schmpt 159–160° erhalten und erwies sich identisch mit Mono-benzoyl-redukton-monoanil.

Um die eventuelle Bildung von Mono-benzoyl-redukton-monoanil bei der Kondensation zu vermeiden wurde dieselbe auf die möglichst schonende Weise folgendermassen ausgeführt.

160 mg Dibenzoyl-redukton wurden in möglichst wenig Chloroform bei 20° gelöst und mit 0.05 ml Anilin versetzt. Nach etwa einer Minute kristallisierte eine Substanz in Form kleiner zitronengelber Stäbchen aus. Das Rohprodukt, 75 mg zeigte den Schmpt 143–144°. Es wurde aus etwas Chloroform umkristallisiert bis zum Schmpt 143,5–144°.

Die Substanz ist leichtlöslich in Alkohol, Methanol und Azeton, löslich in Chloroform, schwerlöslich in Petroleumäther.

Analyse 194:

Gef. C: 73,64 % H: 5,07 % N: 4,93 %.

Diese Analysenwerte stimmen jedoch nicht gut mit dem erwarteten Dibenzoyl-redukton-anil:

$C_{23}H_{17}O_4N$ ber. C: 74,4 % H: 4,6 % N: 3,8 %.

Cocker der diese Zusammensetzung für seine Substanz angibt, hat auch dasselbe Analysenresultat erhalten das mit meinem gut übereinstimmt:

C: 74,0 % H: 5,3 % N: 5,1 %.

Die Analysen stimmen besser überein mit der Bruttoformel

$$C_{35}H_{28}O_6N_2 \text{ bzw. } C_{52}H_{43}O_9N_3.$$

Über die Konstitution der Substanz ist nichts näheres bekannt.

G) Umsetzung der unter B) erhaltenen Substanz mit Essigsäure

250 mg der gelbroten Substanz wurden bei Zimmertemperatur in möglichst wenig Eisessig gelöst, wobei die Lösung sofort einen etwas heller gelben Farbenton annahm. Sie wurde mit etwas Benzol versetzt, wobei eine Substanz in langen gelben Nadeln auskristallisierte die sich mit dem essigsauren Salz des Redukton-dianils identisch erwies. Ausbeute 100 mg. Unter den gleichen Bedingungen wurde das gleiche Salz erhalten wenn die von Eistert und Hasselquist isolierte freie Base des Redukton-dianils mit Essigsäure umgesetzt wurde.

H) Katalytische Hydrierung von Monobenzoylredukton

100 mg Monobenzoylredukton und 10 mg Platinoxyd wurden in 15 ml Alkohol in einer Schüttelapparatur bei Zimmertemperatur hydriert. Das Hydrierungsgemisch wurde nach Aufnahme von 2 Mol H_2 unter vermindertem Druck bis zur Trockne eingeengt. Das Reaktionsprodukt wurde in Äther aufgenommen und vom Pt abfiltriert. Nach dem Vertreiben des Äthers blieb ein kristalliner, aber mit Öl vermischter, Rückstand. Das kristalline Produkt liess sich mit Benzin bei Zimmertemperatur extrahieren und zeigte einen Schmpt von 120–121°. Es ist identisch mit Benzoesäure. Das ölige Produkt war in Wasser unlöslich, also nicht Glycerin, es liess sich nicht zur Kristallisation bringen.

In einem neuen Versuch wurde die Hydrierung nach Aufnahme von 1,5 Mol H_2 abgebrochen. 400 mg Monobenzoylredukton und 23,8 mg Platinoxyd wurden in 50 ml Alkohol bei Zimmertemperatur hydriert. Es zeigte sich gegenüber dem vorherigen Versuch keine Änderung. Es konnte nur Benzoesäure und eine ölige Substanz gewonnen werden. Letztere konnte nicht zur Kristallisation gebracht werden. Der Versuch, vielleicht ein kristallisierbares Hydrazon zu erhalten, schlug fehl. Der Umsatz des Öls mit Phenylhydrazin führte zu einer teerartigen Masse, die nicht zur Kristallisation gebracht werden konnte.

Unter den oben angeführten Versuchsbedingungen konnte kein Monobenzoylglycerin erhalten werden.

Herrn Armin Glaser danke ich bestens für eifrige und geschickte Mitarbeit bei den Versuchen.

Stockholm, Institut für Organisch-Chemische Forschung und Vitamininstitut, Juni 1953.

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Proteolytic activity in sea urchin gametes

III. A study of the proteolytic enzymes of the egg

By GUNNAR LUNDBLAD and INGVAR LUNDBLAD

With 7 figures in the text

During the last few years, eggs and sperms have proved to be interesting sources of proteolytic enzymes for the biochemist. Not only that various types of these enzymes are present in this material, but the properties and varying quantities of the proteolytic enzymes here are of special interest, as eggs and sperms, fertilization and the early development of new individuals are of central biological interest.

For investigations of this kind it is suitable to choose animals, whose eggs and sperms can easily be obtained in satisfying quantities and whose fertilization has been extensively studied. For this reason we have taken eggs and sperms from sea-urchins for our investigations.

In the first papers of this series (1, 2, 3) it was shown that various proteolytic enzymes are present in this material and that the activity of at least one of the enzymes is strongly increased immediately after fertilization.

In the previous investigations the activity was determined viscosimetrically, using gelatin and calculated using HULTIN's formula (4). Although the proteolytic activity is very weak it has been possible to measure it stoichoimetrically by formol titration and this paper gives also the stoichiometric activity of the sea urchin egg proteolytic enzymes.

The investigations in previous works have been made almost exclusively on the enzyme or enzymes with maximum activity near pH 7. In the first and second parts of the present paper the activity at various pH of the different proteolytic enzymes of the egg after various treatment has been measured.

It has been necessary to make several experiments as the properties of the egg material in respect of proteolytic enzymes are very variable, as has been already pointed out (3). The third part of this paper deals with the enzyme with maximum activity at pH 4.3. As the activity of the first mentioned enzyme or enzymes was often very labile and weak or even absent, it was considered suitable first to see whether the enzyme with maximum activity at pH 4.3 was always present. As this proved to be the case, and as the enzyme was fairly stable, it was possible to study it more thoroughly. It was thereby *i.a.* found that the enzyme can hydrolyze 1-benzoylarginine amide, which is one characteristic property of proteolytic enzymes of the cathepsin type.

Experimental

The sea urchin species *Arbacia lixula* and *Paracentrotus lividus* used in the present work were from the Gulf of Naples and the species *Echinocardium cordatum* and *Brissopsis lyrifera* from the West Coast of Sweden. The investigations were carried out at the Zoological Station of Naples during the winter and spring of 1952 and at the Zoological Station of Kristineberg on the Swedish West Coast during the summer of 1952.

Homogenates

The eggs from *Arbacia lixula* were obtained by injection of potassium chloride by the method of TYLER (5) as reported in the previous paper (3), whereas the eggs of *Paracentrotus, Echinocardium* and *Brissopsis* were prepared by taking out the rinsed ovaries. If not otherwise stated the eggs were washed 2–3 times in sea water, and then in isotonic sodium chloride solution 2-3 times. The washing procedure lasted a couple of hours at the temperature of the running aquarian sea water. The eggs were left to settle spontaneously or were carefully centrifuged. The egg material was then suspended in twice its volume of the cold homogenization medium and homogenized in a ground pyrex tube furnished with a well fitting piston. The formation of air bubbles was avoided throughout the preparation, which lasted about 10 minutes. When the material was thoroughly homogenized, which was checked under the microscope, the material was centrifuged in an angle centrifuge at about 8000 rpm (5000 × g).

Lyophilized material

Unfertilized *Paracentrotus* eggs were washed once in sea water, freed from their jelly coats and washed in isotonic sodium chloride. After careful centrifugation the egg material was rapidly frozen and then dried in vacuum without melting. The dried material was suspended in water (2.5 % dry weight) or other medium, frozen, thawed, kept in the cold over night and centrifuged.

Determination of the proteolytic activity

1. *Viscosimetrical.* As reported in previous papers (2, 3) a 4.00 % buffered gelatin solution, containing 0.005 % merthiolate (Lilly) as sterilizing reagent was used. The reaction mixture was made up of a 1.00 ml sample (homogenate or extract) and 3.00 ml gelatin or of a 1.00 ml sample, 0.50 ml reagent and 3.00 ml gelatin. The mixing of sample and gelatin signifies the start. The temperature was 35.5° C throughout the experiments. In some experiments cysteine hydrochloride was added to the sample before mixing with gelatin, the pH of the cysteine solution being adjusted if necessary. In some other experiments cysteine was added to the enzyme-gelatin mixture some time after the start. The activity was measured viscosimetrically (Ostwald viscosimeter) as previously and the values calculated according to HULTIN's formula[1]

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[1] Hultin's formula: $A^{t^\circ} = J \cdot c^2{}_s \cdot \dfrac{d\dfrac{1}{\eta_{sp}}}{d\,t}$

where A = the enzymic acitivity at t° C (units per gram) in the reaction mixture,
J = a factor for the influence of i. a. pH (for most substrates $J = 1$; independent of pH).
c = the concentration of the substrate in the reaction mixture (gram substrate per gram solution),
η_{sp} = specific viscosity and
t = time in seconds

(cf. (2) or (4)). The activity is expressed as A, mμA $= A \times 10^{-9}$. A^{I}: original activity. A^{II}: activity after addition of cysteine. The concentration of cysteine in the mixture of enzyme and reagent is in most cases 0.01 M. It is found in control experiments that a concentration of 0.001 M cysteine gives full activation. Cysteine has no influence upon the viscosity of the gelatin.

2. *Formol titration.* The measurements were carried out by the method of SØRENSEN (6) and NORTHROP (7). Three ml cysteine activated extract (0.01 M), 3.00 ml buffer solution and 6.00 ml of a 6.00 % non-buffered gelatin solution was incubated at each pH. Two samples of 2.00 ml each were taken immediately from this mixture, mixed with 3.00 ml of a 17% formaldehyde solution in order to stop the reaction. Then a 2 ml sample was taken from the incubation mixture for determination of the pH, and the remainder kept for a certain time at 36° C before 2 more samples of 2.00 ml were taken, mixed with 3.00 ml formol, and titrated with 0.01 N sodium hydroxide after addition of 2 drops of phenolphtalein (1.0 % in ethanol).

3. *1-Benzoylarginine amide splitting activity.* In all essential features the determination was carried out by the method of SCHULTZ (8) by mixing 1.00 ml buffer, 0.20 ml neutralized 1-benzoylarginine amide (0.1 M), and 1.00 ml cysteine activated homogenate or extract. One ml of each sample was taken immediately as a control, and the rest incubated for 21 hours at 35.5° C. The determination of the liberated ammonia was made by the Conway technique (9), addition of Nessler's reagent and measurement in a Beckman spectrophotometer at $\lambda = 4300$ Å.

Total nitrogen was determined by means of Kjeldahl combustion and addition of Nessler's reagent. The pH was always checked with a Beckman pH-meter with glass electrode.

Results

Part I. The proteolytic activity in the egg

The activity-pH-curve of an extract form fertilized *Arbacia* eggs reported in a previous paper of this series (2), and determined viscosimetrically with gelatin as substrate, was possibly unreliable because of the corrections necessary due to the variation in specific viscosity of the gelatin at different pH. Extracts from fertilized *Arbacia* eggs were studied, as the activity in extracts from lyophilized unfertilized eggs was too low. In the present work the splitting of gelatin was determined instead by means of formol titration and the results is shown in Fig. 1. This method could not be used from the beginning due to the extremely low activity of the extract, which it was possible to measure only by the sensitive viscosimetrical method. Not until the discovery of the much stronger proteolytic activity, which, as shown by one of the writers (G. L.), sometimes occurs in fresh homogenates, was it possible to determine an activity-pH-curve by means of formol titration.

The results from three different series are shown in Fig. 1. The first (I) was made on a distilled water homogenate from unfertilized *Arbacia* eggs, washed in sea water and isotonic sodium chloride solution in the ordinary way. The viscosimetrical activity of the homogenate was 144 mμA/ml at pH 6.5. For the formol titration the homogenate-gelatin mixture was incubated for 12 hours at 36° C. The second series (II) was made on the same homogenate 2 days later and the incubation time for the formol titration was 21 hours at 36° C. The viscosimetrical activity at pH 6.5 is 156 mμA/ml. The third series (III) was made on another *Arbacia* homogenate prepared as above, and incubated 18 hours at 36° C. The viscosimetrical activity at pH 6.5 = 142 mμA/ml.

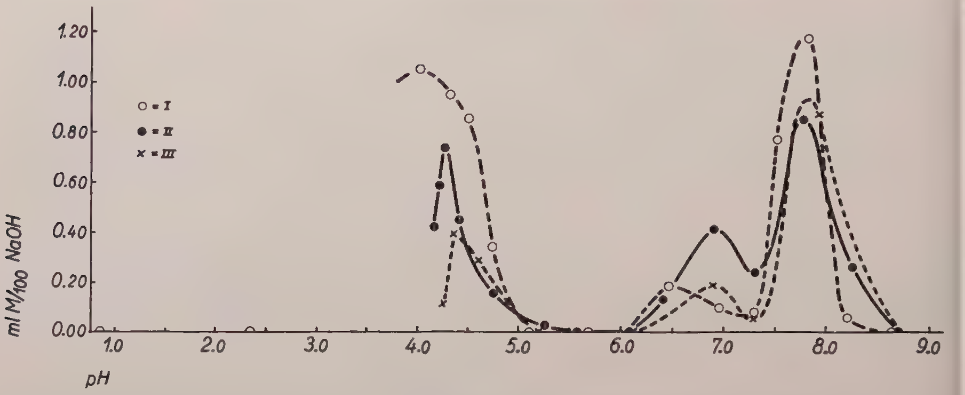

Fig. 1. Activity-pH-curves made on homogenates from fresh unfertilized *Arbacia* eggs. Formol titration.

An equal volume of a 0.01 M neutralized cysteine hydrochloride solution was added to the centrifuged homogenate and this mixture was kept for 15 minutes at + 18°C. Reaction mixture: 3.00 ml buffer solution +3.00 ml activated homogenate +6.00 ml of a 6.00 % unbuffered gelatin solution. Two samples of this mixture were taken immediately after mixing (2.00 ml each) and added to 3.00 ml formol (formaldehyde : water 1 : 1). The rest of the reaction mixture was kept for a certain time at +36°C before the next two samples were taken. The formol-gelatin mixture was titrated with 0.01 M NaOH using phenolphatelein as indicator.

Series I: Distilled water homogenate from unfertilized *Arbacia* eggs. 1.34 mg N/ml.
Series II: The same homogenate stored two days in the cold before measurement.
Series III: The same kind of homogenate made on another batch of eggs. 1.48 mg N/ml.

As can be seen from the figure all the series have three optima at about the same pH values: 4.0–4.4, 6.6–6.9, and 7.8. There is no activity between pH 5.5 and 6.0, a minimum at pH 7.3, and no activity at pH higher than 8.6. Four days later the activity of the homogenate was measured at pH 4.4, 6.5 and 7.6 but activity was found only at pH 4.4 (mμA/ml =24.4, mμA/mg N = 16.6). Addition of cysteine increased the activity at this pH to 144 and 97.4 but there was still no activity at pH 6.5 or 7.6. The appearance of the curves in the figure is in rather good conformity with the activity-pH-curve in a previous work (2). A study of Fig. 3 in

Table 1. The activity of a dialysed homogenate.

Part of the cysteine activated extract studied in series III (Fig. 1) was dialysed against distilled water for 18 hours at + 3°C. Another part of the extract was kept as a control. No precipitation occurred during dialysis. 1.00 ml homogenate was mixed with 3.00 ml gelatin and measured. 1.48 mgN/ml. A^{I}: orginal activity, A^{II}: activity after addition of cysteine 40 min. after the start (ca 0.01 M). pH 6.5.

Exp. No.	Treatment of homogenate	$\frac{m\mu A^{I}}{ml}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{mg\ N}$
1	— — —	0	142	96
2	Kept 18 h:s as control	0	13.2	8.9
3	Dialysed	0	4.6	3.1

that work reveals two optima, one at pH 4.5 and another around pH 6.7 with a minimum near 5.2.

The homogenate studied in Fig. 1, series III was subjected to dialysis and subsequent cysteine treatment and the results are shown in Table 1.

During the dialysis, as well as during the 18 hours' storage, the original activity obtained with cysteine decreased considerably, as shown in Table 1. The control can be reactivated to about 10 %, the dialysed homogenate only to approximately 3 %. Thus twice as much was destroyed in the sample submitted to dialysis as in the control. The extracts were not activated by Ca^{++}.

The effect of calcium and cysteine upon the non-dialysed homogenate was studied also on the enzyme possessing optimal activity at pH 4.3 and the results are shown in Table 2.

Table 2. Activation of an *Arbacia* homogenate with cysteine and calcium.

The same homogenate was studied as in Table 1. 1.00 ml homogenate was incubated with 0.50 ml of the reagent for 15 min. at 18°C and then mixed with 3.00 ml gelatin. pH 4.3. $CaCl_2$ = = 0.003 M. Significance of A^I and A^{II}, se above. Cysteine was added 2 hours after the start.

Exp. No.	Reagent	$\frac{m\mu A^I}{ml}$	$\frac{m\mu A^I}{mg\ N}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{mg\ N}$
4	H_2O	30.5	20.6	87.5	59.2
5	$CaCl_2$	56.0	37.8	56.0	37.8

Calcium has a strong influence almost doubling the activity, but the addition of cysteine has no further effect. Addition of cysteine alone increases the activity.

In a homogenate from *Paracentrotus* eggs it was possible to find enzymatic activity both at pH 4.3 and 7.8. The results of some activity experiments upon this homogenate are shown in Table 3.

Table 3. Some activity experiments on homogenate of *Paracentrotus* eggs.

Paracentrotus eggs were washed 3 times in sea water, in isotonic sodium chloride, homogenized in distilled water, and centrifuged (5000 × g). The nitrogen content was 0.70 mg/ml, pH 6.4. In experiments 6–7, 1.00 ml was mixed with 3.00 ml gelatin and measured. In experiments 8–10, 1.00 ml was incubated with 0.50 ml of the reagent for 30 min. at + 18°C, mixed with 3.00 ml gelatin and measured. Cysteine was added after about 1 hour in experiments 6–7. IBA = o-iodoso benzoic acid.

Exp. No.	Reagent	pH in mixt.	$\frac{m\mu A^I}{ml}$	$\frac{m\mu A^I}{mg\ N}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{mg\ N}$
6	— — —	4.3	120	172	120	172
7	— — —	7.4	33	47	0	0
8	H_2O	7.8	80	114	—	—
9	$CaCl_2$ 0.03 M	7.8	80	114	—	—
10	IBA 0.001 M	7.8	80	114	—	—

The experiments in Table 3 show that distilled water homogenate has a rather good activity at pH 4.3, which is not increased by cysteine. The activity at pH 7.8 is lower but 2–3 times as high as the activity at pH 7.4. The proteolytic activity at pH 7.8 is not affected by calcium or the sulphydryl inhibitor IBA. The activity at pH 7.4, 33 mμA/ml, approaches zero in an hour and cannot be restored by cysteine.

Activity of fertilized *Paracentrotus* eggs

As a complement to previous studies the proteolytic activity of two fertilized stages of *Paracentrotus* were investigated at pH 6.5; the results are shown in Table 4. It was necessary to add cysteine in order to obtain any activity. The activity was several times higher in the extracts from the fertilized stages than in that from the unfertilized material. It was not possible to test the activity at pH 4.3 as only small amounts of the material were available.

The activity at pH 4.3 and 6.4 of some other extracts from *Paracentrotus* (see Table 5), from unfertilized eggs and from a stage taken 8 min. after fertilization was studied. In these extracts however no activity could be detected at pH 6.4, and the activity at pH 4.3 was not increased after fertilization. Cysteine, added 40 min. after the start of the measurement, had no influence on the activity.

Table 4. Activity of unfertilized eggs and 2 stages of fertilized *Paracentrotus* eggs.

Equivalent amounts of *Paracentrotus* eggs from unfertilized and 2 fertilized stages were rapidly frozen down, thawed and centrifuged. 1.00 ml of the extract was mixed with 3.00 ml gelatin and measured at pH 6.5. Cysteine was added 1 hour after the start.

Exp. No.	Stage	$\frac{\text{mg N}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{mg N}}$
11	Unfertilized	0.76	0	2.8	3.7
12	Blastula (12 hours) .	0.59	0	9.6	16.3
13	Early plutei (42 hours)	0.31	0	16.8	54.2

Table 5. Activity at pH 4.3 of unfertilized and fertilized *Paracentrotus* eggs.

Equal amounts of an unfertilized and a fertilized stage of jelly coat-free, washed *Paracentrotus* eggs, were taken 8 min. after the fertilization and shell frozen, thawed and distilled water added. The material was again frozen, thawed, kept 1 hour at + 3° C, centrifuged (5000 × g) and measured. Equivalent parts were then acidified to pH 4.3 and centrifuged. The first extracts were measured at pH 6.4 but found to be inactive. The acidified extracts, measured at pH 4.3 were active.

Exp. No.	Stage	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{ml}}$
14	Unfertilized	500	500
15	Fertilized 8 min.	450	450

The influence of extracts from different stages of *Arbacia* eggs upon a papain solution

Several experiments on the activity of homogenates or extracts when incubated with a weak papain solution (no cysteine added) were reported in part II of this series of investigations (3). This procedure suggested by MYRBÄCK (10–12), revealed that the papain itself became more active during its action on the egg homogenate or extract. Thus papain could be used as an agent (medium) for testing the effect on the material of different methods of treatment. A series of experiments are shown in Fig. 2 in the present work. Unfertilized eggs and those taken 5, 10 and 40 minutes after fertilization were extracted, the extracts incubated with papain, and the gelatin splitting activity measured. As seen in the figure extract from unfertilized eggs had the highest activity which then decreased in the later stages of fertilized eggs. The original proteolytic activity of these extracts was zero (unfertilized) and the other stages very low (0.5–1.4 mμA/ml).

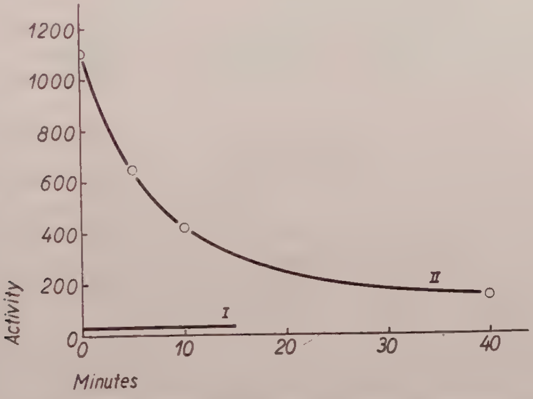

Fig. 2. The influence of extracts from different stages of *Arbacia* eggs upon a papain solution.

Lyophilized *Arbacia* eggs from different stages were extracted (2.5 % dry weight) in distilled water, frozen, thawed, kept for 16 hours at +3° C and centrifuged. Unfertilized eggs and eggs taken 5, 10 and 40 minutes after fertilization were extracted. The nitrogen content was in the same order: 1.06, 1.00, 1.15 and 1.20 mg/ml. The lyophilized egg material was stored for 1 year at —20° C. The activity is expressed as viscosimetrical units (cf. HULTIN (4)) mμA/mg N on the ordinate and time after fertilization is on the abscissa.

Reaction mixture: 1.00 ml papain solution (papain powder Tanganyika, 1 % dry weight) was incubated with 0.50 ml H_2O or extract for 30 minutes at +20° C, mixed with 3.00 ml gelatin and measured. pH 6.7 at all measurements.

I: Activity of papain incubated with distilled water.

II: Activity of papain incubated with extracts from 0, 5, 10 and 40 minutes' stages.

Proteolytic activity in *Echinocardium* and *Brissopsis*

The activity in a homogenate from unfertilized eggs of *Echinocardium cordatum* was measured at pH 4.4 and 6.6; the values are shown in Table 6. No activity could be found at pH 6.6, either with or without cysteine; a rather strong activity at pH 4.4 was increased more than three times by addition of cysteine.

The results reported in Table 7 are from an extract of oocytes of *Brissopsis lyrifera.* The activity of unripe eggs, oocytes, was a matter of considerable interest and as it is possible to find gonades containing about 95 % oocytes in the sea urchin species *Brissopsis*, this was chosen for study. The activity at pH 4.4 and 6.6 was determined. No activity could be found at pH 6.6, either with or without cysteine; at pH 4.4 there was a rather strong activity which was increased more than three times by addition of cysteine.

Table 6. Proteolytic activity in unfertilized eggs of *Echinocardium cordatum*

Extract I: Jelly coat free eggs, washed in sea water were suspended in distilled water, kept in the cold over night and centrifuged. Extract II: A part of the extract (I) was acidified to pH 4.4 and centrifuged in order to remove the resulting precipitate. 1.00 ml of the extract was mixed with 3.00 ml gelatin and measured.

Exp. No.	Extract	pH	mμA/ml
16	I	6.6	0
17	II cysteine	4.4	400
18	II	4.4	120

Table 7. Proteolytic activity in extract of oocytes from *Brissopsis lyrifera*.

Egg material from *Brissopsis*, containing ca. 95 % oocytes, was washed in sea water three times and then suspended in three times the sedimented egg volume of distilled water, frozen, thawed, kept in the cold for 6 hours and centrifuged (I). A part of (I) was acidified to pH 4.4 and centrifuged (II). A part of (II) was dialysed in the cold against distilled water for 22 hours (II D). Another part of (II) was kept in the cold for the same time (II C). All measurements in experiments 19–24 were made at pH 4.4.

Exp. No.	Extract	mμA/ml
19	II	870
20	II cysteine	900
21	II C	860
22	II C cysteine	880
23	II D	70
24	II D cysteine	1 300

The experiments in Table 7 showed that the activity of the untreated oocyte extract is very high, that it is increased very little by cysteine and that the activity is almost unchanged after standing for 22 hours in the cold. Dialysis destroys more than 90 % of the activity; the remaining activity is however increased to a value about 50 % higher than that in the control by addition of cysteine. This may indicate the presence of an inhibitor, removed by the dialysis.

The influence of Soya Bean Inhibitor (SBI) upon extract (II) was investigated and it was shown to decrease the activity by about 25 % after cysteine addition. 1 mg SBI added to 1.00 ml of extract (II), incubated for 30 min. at +20° C and mixed with 3.00 ml gelatin. Activity after the addition of cysteine = 600 mμA/ml (in untreated extract = 800 mμA/ml). Without cysteine both samples were inactive.

Part II. Homogenization and extraction of *Arbacia* and *Paracentrotus* egg in different media

In a previous paper (3) the effect of some substances acting on the egg material during the homogenization was studied and the influence of the same substances upon the activity of centrifuged homogenates was measured. Some preliminary experiments on separated ("fractionated") homogenization in different media were also made in that paper. More extensive experiments on homogenization in different media and homogenization in buffered solution, with subsequent extraction in the same or in another kind of medium were carried out in this work and the results are shown in Tables 8–15.

Fractionated homogenization and extraction of *Arbacia* eggs

In Table 25 in the previous paper (3) the results of some experiments to locate the site of the proteolytic enzymes of the egg are shown. This was also done in the present investigation with the exception that the extraction for 1 hour at 18°C described there was instead made in the cold, with subsequent extraction of the residue by means of freezing and thawing. The results are shown in Table 8.

Table 8. Separated homogenization of *Arbacia* eggs in non-buffering media with subsequent extraction.

Arbacia eggs were washed twice in sea water and twice in isotonic sodium chloride. The eggs lost their jelly coats spontaneously during these procedures and sedimented rapidly. The material was homogenized in isotonic sodium chloride and centrifuged. The supernatant liquid is called A (pH 5.8). The sediment was suspended in distilled water, kept for $\frac{1}{2}$ hour at + 3° and then centrifuged. This supernatant liquid was called B. The sediment was suspended in distilled water, frozen, thawed up, kept over night at + 3°C and centrifuged. The supernatant liquid was called C (pH 5.7). A part of A was acidified to pH 4.3 and the resulting precipitate centrifuged. The supernatant liquid was called A 2. 1.00 ml of the extract was mixed with 3.00 ml gelatin and measured. Cysteine was added after 1 hour in experiments 27, 30, 31 and after 30 minutes in experiment 28.

Exp. No.	Preparation	$\frac{\text{mg N}}{\text{ml}}$	pH in mixt.	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{mg N}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{mg N}}$
25	A	2.82	7.6	0	0	—	—
26	»	2.82	4.3	Strong precipitation		—	—
27	B	1.95	7.2	0	0	0	0
28	»	1.95	4.3	450	230	339	174
29	A 2 . . .	1.04	4.3	19.5	18.7	—	—
30	C	2.25	7.4	0	0	1.0	0.45
31	»	2.25	4.3	0	0	165	73.4

The results in Table 8 show that in the sodium chloride homogenate the activity could not be measured because of the precipitates formed. After acidification and centrifugation of A the extract A 2 was measured at pH 4.3 and was found to be active. The extract B, made by short extraction of the sediment from the homogenate, was inactive at pH 7.2 and could not be activated by means of cysteine, but possessed a very strong activity at pH 4.3 which, however, it was not possible to increase with cysteine. In the extract C, prepared from the sediment of B extraction, there was no activity either at pH 7.4 or 4.3, but after addition of cysteine very low activity was detected at pH 7.2 but strong activity at pH 4.3. These experiments show that by careful homogenization, as in isotonic solution, only a part of the enzymic activity appears, but under the influence of distilled water in the cold the granular bound enzyme passes into solution in a much higher concentration, and when the remainder is frozen and thawed still greater part passes into solution. The precipitation which occurred with A and which prevented measurement is unusual. That effect was not found in an earlier investigation, where the activity was measured at pH 6.6 (Cf. Table 25 (3)).

Homogenization in water and glycerol

Mixtures of glycerol and water are long since known to be good solvents for certain enzymes in extraction and homogenization procedures. (10, 11). They were tried with negative results at the beginning of the investigations. However glycerol was again tried later under more carefully controlled conditions and the results are shown in Tables 9 and 10.

Table 9. Homogenization of unfertilized fresh *Arbacia* eggs in water or glycerol.

Eggs from *Arbacia* (KCl injection, spooning in sea water) were washed three times in sea water, and twice in isotonic sodium chloride. After careful centrifugation the egg material was divided in two parts, which were homogenized A) in distilled water and B) in 30 % glycerol in water. After centrifugation 1.00 ml of each homogenate was mixed with 3.00 ml of the gelatin solution and measured. pH 6.5. Cysteine added 45 minutes after the start. A^I and A^{II} as in Table 1.

Exp. No.	mg N / ml	Homogenization medium	$m\mu A^I$ / ml	$m\mu A^I$ / mg N	$m\mu A^{II}$ / ml	$m\mu A^{II}$ / mg N
32	1.24	H_2O	94	76	504	407
33	—	Glycerol	0	—	2.7	—

As can be seen from Table 9 homogenization in water gave a good activity which could be increased considerably by cysteine. The glycerol homogenate had no activity in itself and with cysteine only a weak activity was obtained.

The extracts described in Table 10 were all measured at pH 4.3. The extracts A, B, AR, and BR were measured at pH 7.3 but were all inactive. It was impossible to measure them at pH 4.3 due to the formation of precipitates and the four extracts were therefore acidified and centrifuged before being measured at pH 4.3. Homogenization in glycerol gave only about 5 % volume for volume of the actitvity of the water homogenate. The low nitrogen content of the glycerol homogenate is noteworthy. Cysteine had no influence upon the activity. In the frozen and thawed aqueous extract of the undissolved residue (exp. 36) the activity is extremely high, at the start of the measurement, but after addition of cysteine the activity decreases considerably. In the corresponding glycerol extract (BRS) the original activity is ca 20 % of the aqueous extract but in this extract cysteine has an activating effect.

Table 10. Homogenization and extraction of *Arbacia* eggs in distilled water or glycerol.

Arbacia eggs were washed in sea water, the jelly coats removed, the egg material washed again in sea water and twice in isotonic NaCl. The egg material was divided in 2 parts: A) homogenized in distilled water and B) homogenized in 30 % glycerol in water and then centrifuged (5000 × g). These supernatant liquids are A and B (pH 5.6). The sediments from A and B were suspended in distilled water and aqueous glycerol, respectively frozen, thawed, kept at + 3°C for 3 hours and centrifuged. These extracts are AR and BR (pH 5.6). Parts of these extracts were acidified to pH 4.4 and the precipitates centrifuged. The supernatant liquids are called ARS and BRS. Parts of A and B were also treated in the same way, they are called AS and BS. 1.00 ml extract was mixed with 3.00 ml gelatin and measured at pH 4.3 or 7.3. A^{II}: cysteine is added about 20 minutes after the start. Equivalent volumes were used during the whole preparation.

Exp. No.	Preparation	$\frac{\text{mg N}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{mg N}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{mg N}}$
34	AS	1.65	265	161	265	161
35	BS	0.03	14	467	14	467
36	ARS	1.12	1 310	1 170	640	572
37	BRS	3.04	245	80.6	300	98.7

Homogenization in buffered solutions

The sensitivity of several enzymes to the influence of pH makes it desirable to extract an enzyme at the pH value of the optimal activity or stability of that enzyme. This is carried out in a solution with regulated pH. The technique is extensively used. In material containing two or more enzymes with different pH optima, an extraction at one of these pH values may be followed by a second at the other pH. By means of this technique it is possible to obtain only one or mainly one of the enzymes in the active state in solution in each extract. In most cases, however, the material is divided in two parts, each part being extracted or homogenized in the actual buffered solution. In the present work the method was also used for homogenization in an attempt to protect the unstable "neutral" enzyme from the influence of the strong enzyme of catheptic type, and from inhibiting factors which may be present.

The results of homogenization of *Arbacia* eggs in two different buffers are shown in Table 11. In the next table this operation is followed by extraction of the undissolved part in the same type of buffer. The result of homogenization of *Arbacia* eggs in buffered solutions in the presence of cysteine, with subsequent extraction in buffers without cysteine are shown in Table 13. In Table 14 the same kind of extraction and homogenization was employed as in Table 12, but *Paracentrotus* eggs were used.

As can be seen in Table 11 the activity at pH 4.4 is much stronger in the citrate homogenate than in the imidazole homogenate. The pH-values of the homogenate-gelatin mixture differed somewhat from the pH-values of the chosen gelatin solutions because of the influence of the buffered homogenates. Thus the activity was not measured at the maximal values of the pH curve. It is however likely that homogenization at the neutral point causes higher activity at pH around 6.6 than the citrate homogenization. It is peculiar that the activity is stronger at pH 5.1 than 4.4. The absence of activity at pH 7.4 close to pH 6.9 where a very high activity is found is also noticeable. No cysteine was used in this homogenate.

Table 11.

Arbacia eggs were washed 3 times in sea water and then twice in isotonic sodium chloride. After centrifugation the egg material was divided in two parts, A and B. A was homogenized in citrate buffer at pH 4.3, and B was homogenized in imidazole buffer at pH 7.2. 1.00 ml homogenate was mixed with 3.00 ml of gelatin buffered to the corresponding pH and measured.

Exp. No.	Preparation	$\frac{\text{mg N}}{\text{ml}}$	pH of gelatin	pH in mixt.	$\frac{\text{m}\mu\text{A}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}}{\text{mg N}}$
38	A	1.36	4.3	4.4	231	170
39	A	1.36	6.6	5.1	432	317
40	A	1.36	7.8	6.1	97.2	71.5
41	B	3.60	4.3	4.4	77.8	21.6
42	B	3.60	6.6	6.9	234	65.0
43	B	3.60	7.8	7.4	0	0

Table 12. Homogenization of *Arbacia* eggs in buffers followed by buffered extraction.

Arbacia eggs were washed twice in sea water, the jelly coats removed by means of acidification, and the egg material thereupon washed twice in sea water and once in isotonic sodium chloride. This is called Iso. Med. The material was divided into 2 parts A and B and homogenized: A) in citrate buffer at pH 4.3 and B) in imidazole buffer at pH 7.2, and then centrifuged. The supernatant liquids were called Sup. A and Sup. B. The sediments from A and B were suspended in the same volume of the corresponding buffers, frozen, thawed, kept overnight at + 3°C and then centrifuged. They are called AR and BR. The isotonic medium was slightly coloured because of partially cytolysis. Cysteine was added 1 hour after the start in experiments 44–45, and after 20 minutes in experiments 46–47. The activity of the A-homogenates was measured at pH 4.3, and that of the B-homogenates at pH 6.7, but as only the acid homogenates were active only these results are reported in the table. 1.00 ml of homogenate was mixed with 3.00 ml gelatin and maesured at pH 4.3.

Exp. No.	Preparation	$\frac{\text{mg N}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{mg N}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{mg N}}$
44	Sup. A	1.16	19.2	16.6	57.0	34.2
45	Iso. Med. (supernat.)	0.05	0	0	1.6	32.0
46	AR	4.08	1 090	267	1 090	267
47	AR + JC	4.08	1 110	272	1 110	272

Table 12 shows how the undissolved part of the homogenate, when further treated by freezing and thawing in the same kind of buffer, gives an extract (AR) containing 3.5 times as much nitrogen, and possessing about 15 times stronger activity per mg nitrogen. The freezing and thawing seems to have liberated the enzyme fixed to a large extent to the granular parts of the cytoplasm than freely dissolved in the cytoplasm. In this extract the enzyme seems to be at its optimal activity with respect to functional sulphydryl groups. A very small amount of the enzyme had leaked out into the medium, which however must be activated with cysteine. Five minutes treatment at +18°C with an *Arbacia* jelly coat (JC) solution has no effect upon the activity.

As jelly coats from sea urchin eggs mainly consist of sulphuric acid esters of polysaccharides, the influence upon fertilization of a similar substance, heparin, has been subject to several investigations. The effect of heparin on homogenates and extracts from unfertilized eggs was studied by the writer at pH 4.3 and 6.7. One ml of an egg preparation was incubated with 0.50 ml 0.1–0.2 % heparin, BWD, for 30 minutes at 20° C and then mixed with 3.00 ml gelatin and measured. All preparations were sufficiently active without addition of cysteine. No effect was found.

Table 13. Homogenization of *Arbacia* eggs in buffers in presence of cysteine, with subsequent extraction.

Arbacia eggs were washed twice in sea water and twice in isotonic sodium chloride. After centrifugation a part of the material was homogenized in citrate buffer containing 0.005 M cysteine. The centrifuged solution from the homogenate was called A. The sediment was suspended in the same volume of citrate buffer (no cysteine), frozen, thawed, kept for 3 hours at + 3° C and centrifuged. This extract was called AR. Analogous preparations in imidazole buffers (pH 6.6) were called B and BR. 1.00 ml homogenate or extract was mixed with 3.00 ml gelatin and measured at pH 4.3.

Exp. No.	Preparation	$\frac{\text{mg N}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}}{\text{mg N}}$
48	A	1.32	122	92.5
49	AR	4.00	896	224

These experiments in Table 13 indicate that the enzyme is mainly bound to granules in the cytoplasm. The preparations B and BR were inactive at the pH of that buffer (6.6).

Table 14. Homogenization of *Paracentrotus* eggs in buffers, with subsequent extraction.

Paracentrotus eggs were washed twice in sea water and twice in isotonic sodium chloride. The material was divided in 2 parts and homogenized: A in citrate pH 4.2 and B in imidazole buffer (pH 7.2). After centrifugation (5000 × g) the supernatant liquids were collected and called A and B. The sediments from A and B were suspended in the same volumes of the respective buffers, frozen, thawed, kept at + 3° C for 2 hours and centrifuged. The supernatant liquids are called AR and BR respectively. 1.00 ml extract was measured after being mixed with 3.00 ml gelatin. Cysteine was added 30 minutes after the start.

Exp. No.	Preparation	$\frac{\text{mg N}}{\text{ml}}$	pH of extr.	pH in mixt.	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{I}}}{\text{mg N}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{ml}}$	$\frac{\text{m}\mu\text{A}^{\text{II}}}{\text{mg N}}$
50	A	7.9	4.3	4.3	190	24.0	190	24.0
51	B	2.27	6.5	6.5	102	45.0	9.6	4.23
52	AR	0.59	4.3	4.3	0.5	0.85	340	576
53	BR	1.73	7.1	7.4	0	0	0	0

Table 14 shows that activity at pH 4.3 of this acid homogenate is rather strong, but independent of cysteine. The neutral homogenate B had a considerable activity during the first 30 minutes, which activity could not be increased by cysteine. In the acid extract from the sediment of A there are only traces of activity which, however, becomes very strong after addition of cysteine about 1 hour after the mixing of the extract and gelatin. In the neutral extract of the sediment of B no activity was demonstrabel, possibly due to the unfavourable pH.

Homogenization in sucrose

The experiments in Table 15 were performed during a period when no activity on the whole could be found around pH 7. Thus the lack of activity in the sucrose at this pH cannot surely depend on this medium. The results are however of some interest. The homogenization was carried out in 0.25 molar isotonic sucrose (cf. (13)) in which many enzymes seem to be stable. Non-buffered sucrose solution was used as homogenizing medium as well as citrate buffered sucrose.

Table 15. Homogenization in non-buffered and in buffered sucrose solution.

Preparation I:

Arbacia eggs, washed in sea water and isotonic sodium chloride in the usual way were homogenized in 0.25 M sucrose solution and centrifuged. 1.00 ml of this solution was mixed with 3.00 ml gelatin and measured. 2.27 mgN/ml, pH of I = 5.8. Cysteine was added 30 minutes after the start.

Preparation II:

Arbacia eggs treated as above were homogenized in 0.25 M sucrose, dissolved in citrate at pH 7.25. A part of this homogenate (II) was acidified to pH 4.1 and the resultant precipitate centrifuged. The clear solution was called II S. 0.46 mgN/ml. Cysteine was added 60 minutes after the start.

Exp. No.	Preparation	pH of gelatin	pH in mixt.	$\frac{m\mu A^{I}}{ml}$	$\frac{m\mu A^{I}}{mg\ N}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{mg\ N}$
54	I	4.3	4.3	1 330	586	642	284
55	I	7.8	7.2	0	0	0	0
56	II	7.8	7.6	0	0	0	0
57	II S	7.8	7.4	0	0	0	0
58	II S	4.3	4.3	105	228	317	689

From Table 15 it can be seen that the activity in the fresh non-buffered homogenate is extremely strong; however, the activity decreases to zero in about 10 minutes, but after addition of cysteine 50 % of the activity is restored. At pH 7.2 there was no activity. The original intention was to carry out the measurement at pH 7.8 but the pH of the enzyme-gelatin mixture was found to be 7.2, which is near the minimum according to the activity-pH-curve in Fig. 1. As seen in the table the buffered homogenate has activity only at pH 4.3, which is increased after the addition of cysteine.

Part III. The catheptic enzyme

As mentioned in the introduction, the fact that proteolytic enzyme possessing optimal activity around pH 4.4 was always found in homogenates and extracts made it possible to study it more in detail. Another reason for studying the enzyme at this particular pH is that it can most likely be classified as of cathepsin II type as will be shown later in this part of the work.

Experiments on reactivation of the enzyme.

The results of Part I and II in this paper showed that the "acid" enzyme could be strongly activated by means of cysteine. This may indicate that it belongs to the thiol type of proteolytic enzymes. If this is so, sulphydryl inhibitors must inactivate the enzyme and if the action of the inhibitor in question is reversible, it should be possible to restore the proteolytic activity more or less by means of cysteine, glutathion etc. In Tables 16—18 some experiments on activation, inhibition and reactivation of this enzyme are summarised.

Table 16. Experiments on reactivation of a homogenate.

The homogenate in series III (Fig. 1) was studied. 1.00 ml was incubated with reagent I for 30 minutes and then with reagent II for another 30 minutes (in all 60 minutes), mixed with 3.00 ml of gelatin solution and measured at 35.5° C at pH 4.4. IBA = 0.001 M o-iodosobenzoic acid. Cysteine, HCl neutralized = 0.01 M. Glutathion = 0.01 M. pH at incubation = 5.8.

Exp. No.	Reagent I	Vol.	Reagent II	Vol.	mμA / ml	mμA / mg N
59	H_2O	0.50	—	—	11.2	7.6
60	»	0.25	IBA 30 minutes	0.25	0	0
61	IBA	0.25	Cysteine	0.25	97.1	65.8
62	»	0.25	Glutathion	0.25	160	108
63	H_2O	0.25	»	0.25	97.2	65.8

The experiments in Table 16 show that IBA inactivates completely, and that the activity is fully restored by cysteine and still further increased by glutathion. This may indicate that pretreatment with IBA blocks an inhibitor which is reactivated to a lower degree than the enzyme.

Table 17. Experiments on reactivation of a homogenate.

The same homogenate as in Table 16 treated in the same way 3 days later, with the same type of reagents. pH 4.3.

Exp. No.	Reagent I	Vol.	Reagent II	Vol.	mμA / ml	mμA / mg N
64	H_2O	0.50	—	—	6.9	4.7
65	IBA	0.25	H_2O	0.25	0	0
66	»	0.25	Cysteine	0.25	90.6	61.3
67	H_2O	0.25	»	0.25	93.4	63.0
68	Cysteine	0.25	IBA	0.25	133	89.9

The experiments in Table 17 show that cysteine restores fully the activity destroyed by IBA and that pretreatment with cysteine followed by addition of IBA gives a higher activity than cysteine alone.

Table 18. Experiments on reactivation of a homogenate.

Arbacia eggs were washed 3 times in sea water and twice in isotonic sodium chloride, homogenized in citrate buffer (pH 4.3) and centrifuged. The homogenate was treated in the same way as that in Table 16. IBA = $M \times 10^{-4}$, pH 4.3, 1.36 mgN/ml.

Exp. No.	Reagent I	Reagent II	mμA/ml	mμA/mg N
69	Cysteine	H_2O	258	190
70	IBA	Cysteine	265	195
71	Cysteine	IBA	428	314

The experiments in Table 18 also show that the activity increases considerably when IBA is added to cysteine pretreated homogenate.

In conclusion, the results in Tables 16, 17 and 18 seem to support the theory that there is present in the homogenate an inhibitor which like the enzyme is blocked by SH inhibitors but to a lower degree of reversibility than the enzyme. Thus treatment of the homogenate with both an SH inhibiting and an SH activating reagent gives stronger activity than an activating reagent alone.

The effect of sodium periodate on the enzyme.

The results of treatment of the enzyme with an oxidizing reagent, sodium periodate, are shown in Tables 19 and 20. The inhibiting effect of this reagent appears to be of a different type than that just reported, and probably does not affect the inhibitor in the same way as, for example, o-iodosobenzoic acid. The influence of p-chloromercuribenzoate is also shown in Table 19.

Table 19. Activity experiments on an acetate homogenate.

Arbacia eggs were washed once in sea water, the jelly coats removed by acidification, the egg material then washed again in sea water and twice in isotonic NaCl. The material was homogenized in acetate buffer (pH. 4.5) and centrifuged. 3.15 mgN/ml. 1.00 ml was incubated with 0.50 ml agent for 60 minutes at +20° C, mixed with 3.00 ml gelatin and measured. pH 4.3. CMB: p-chloromercuribenzoate.

Exp. No.	Reagent	mμA/ml	mμA/mg N
72	H_2O	248	78.7
73	CMB $M \cdot 10^{-5}$	144	45.7
74	$NaIO_4$ $M \cdot 10^{-2}$	19.2 (272)	6.1 (86.4)

The results in Table 19 show that p-chloromercuribenzoate destroyed more than 40 % and sodium periodate more than 90 % of the activity. In the last experiment the activity was restored by means of cysteine (the figures in the paranthesis), added 1 hour after the start.

Table 20. Experiments on oxidation of homogenate.

In these experiments 1.00 ml of homogenate was first incubated with 0.25 ml of reagent I for 30 minutes at +20 °C and after that 0.25 ml of reagent II was added and the mixture was incubated for further 30 minutes at the same temperature, whereupon 3.00 ml gelatin was added and the activity measured. A^{I} = the activity of the mixture during the first hour. A^{II} = the activity in the mixture after addition of cysteine (0.01 M), 1 hour after the start. pH 4.3 at all measurements.

Exp. No.	Reagent I	Reagent II	$\frac{m\mu A^{I}}{ml}$	$\frac{m\mu A^{I}}{mg\ N}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{mg\ N}$
75	Cysteine 0.01 N	H_2O	237	75.2	—	—
76	$NaIO_4$ 0.01 N	Cysteine 0.01 N	13	4.1	40	12.7
77	» 0.01 N	H_2O	0	0	0	0
78	Cysteine 0.01 N	$NaIO_4$ 0.01 N	0	0	165	52.4

It can be seen in Table 20, exp. 77, that $NaIO_4$ causes complete inactivation when cysteine is not added as reagent II and that further addition of cysteine to the gelatin mixture has no effect. When however cysteine (as reagent II) is added before mixing with gelatin (exp. 76) about 5 % of the activity (13 mμA/ml) remains and after addition of cysteine to the gelatin-enzyme mixture more than 15 % of the activity can be found. When the homogenate is first treated with cysteine the situation is very different (exp. 78); $NaIO_4$ causes complete inactivation but when cysteine is added during the measurement the activity is now restored to about 70 %. Thus the activity and protecting effect of cysteine is greatest when it is added to the homogenate before another reagent, less when it is added as reagent II and least when it is added to the gelatin-homogenate mixture.

Effects of calcium

In a previous paper (3) the effect of calcium upon some enzymes and upon other biochemical processes was discussed. Several experiments concerning the influence of calcium, carried out in that work, seemed to indicate that the concentration of calcium was of importance. The experiments shown in Table 21 in the present work were made in order to determine the influence of the concentration calcium upon its activating effect. But, as calcium is known to have both an activating and stabilizing effect, the activity was measured and calculated not only at the start but also after some time.

From Table 21 it will be seen that the proteolytic activity increases when calcium is added in an appropriate concentration and that higher concentrations of calcium maintain a part of the activity, which in the untreated homogenate rapidly disappears. Cysteine gives no activation but seems to prevent rapid inactivation of the enzyme. It is of interest to compare these results with those of other authors.

The fundamental role of calcium for activation and stabilization of some bacterial proteinases is reported by **Goring** (14). **Sugawara** (15) found that Ca ions in low concentrations activated the hatching enzyme but in high concentrations inhibited it. **Muggleton** and **Webb** (16) reported that calcium activates a proteinase from soil *Actinomyces*, but that 5 times the optimal calcium concentration completely inhibits the activity.

Table 21. The influence of calcium upon a homogenate.

Arbacia eggs were washed twice in sea water and twice in isotonic sodium chloride, homogenize in citrate buffer at pH 4.3 and centrifuged. 1.68 mgN/ml. 1.00 ml was incubated with 0.50 c the reagent for 30 minutes at + 18°C. pH 4.3.

Exp. No.	Reagent	Conc. in incub. mixture	Activity at the start		Activity after about 45 min.	
			mμA / ml	mμA / mg N	mμA / ml	mμA / mg N
79	H_2O	—	238	142	0	0
80	$CaCl_2$	0.1 M	238	142	110	65.5
81	»	0.01 M	618	368	187	111
82	»	0.001 M	373	222	0	0
83	Cysteine	0.01 M	244	145	244	145

Activity as a function of enzyme or substrate concentration

The homogenate which was studied in Table 21 was further investigated concerning the relationship between enzyme concentration and activity. The results are shown in Fig. 3, from which it can be seen that the activity is proportional to the enzyme concentration except at very low concentration. Thus at the enzyme concentrations used in the present work the activity is proportional to the concentration of the enzyme.

In such experiments, where an enzyme solution (extract or homogenate) is treated with a certain volume of a reagent, both the substrate concentration and the enzyme concentration are changed and consequently it is possible that the activity might

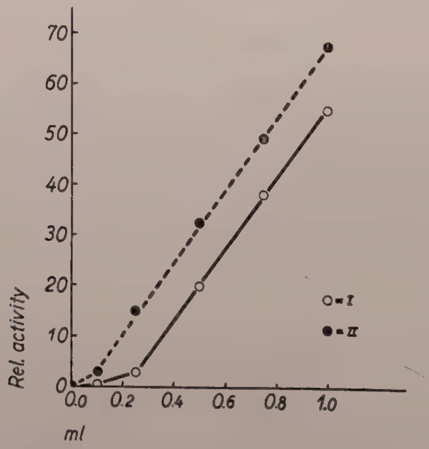

Fig. 3. Activity as function of enzyme concentration.

Unfertilized *Arbacia* eggs were washed twice in sea water and twice in isotonic sodium chloride. After homogenization in citrate buffer, pH 4.3, the material was centrifuged. 1.70 mg N/ml. Different volumes of homogenate diluted with distilled water to a total volume of 1.00 ml were mixed with 3.00 ml gelatin and measured at pH 4.3. Curve I is untreated homogenate and curve II is cysteine treated (0.01 M) homogenate.

be changed. The ordinary substrate concentration in the final mixture is 3.00 % but 2.67 % in those cases in which 0.50 ml of a reagent is added. As an example the following results may be reported. Activity for 1.00 ml enzyme solution + 3.00 ml gelatin solution (4.00 %) = 570 mμA/ml. Activity for 1.00 ml enzyme solution + 0.50 ml H_2O + 3.00 ml gelatin = 590 mμA/ml. Both were double samples in good agreement The enzyme solution was a citrate buffered extract from lyophilized unfertilized *Paracentrotus* eggs (pH 4.5).

Fractionation of *Arbacia* egg extracts by means of the salting out method

One of the most important methods known for the separation proteins is by means of salting out. Generally a saturated solution of ammonium sulphate is slowly mixed in the cold with a protein (enzyme) solution at fixed pH, which ought to be near the isoelectrical point of the actual enzyme, or in case this is not known, near the pH optimum for stability or activity. In Tables 22 an 23 some experiments are reported which were designed to disclose in which fraction the enzyme is to be found, and to separate the enzyme and possibly the inhibitor which is present.

Table 22. Fraction of extracts by means of ammonium sulphate.

Saturated ammonium sulphate was added in portions, at + 3° C, to 50 % saturation at pH 4.5 to a certain volume of the extract studied in Table 12. A slight opalescence occurred at about 25 % saturation. This opalescence became stronger at 50 % saturation but only a very small amount of a precipitate was collected by centrifuging. The clear supernatant liquid was called Sup. 50. To a part of this was added ammonium sulphate to 75 % saturation. A precipitate was then formed which on centrifugation formed a surface layer. This precipitate called Prec. 75 was dissolved in distilled water to the original volume. The clear solution was called Sup. 75. 1.00 ml of the extract to be measured was mixed with 3.00 ml gelatin. In the table both the activities obtained on measurement and those calculated upon the original volume of the extract are given. pH 4.3. Cysteine was added 1 hour after the start.

Exp. No.	Preparation	$\frac{m\mu A^{I}}{ml}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{ml}$ orig. vol.
84	Extract	312	312	312
85	Sup. 50	0	950	1 900
86	Sup. 75	0	330	1 320
87	Prec. 75	0	742	742

It is evident from Table 22 that there is no activity in the absence of cysteine in the treated extract. The activity of the extract when first measured was, as can be seen in Table 12, about 1100 mμA. The greatest part of the enzyme seems to occur in the supernatant liquid at 50 % ammonium sulphate saturation. This activity is 1900 mμA per ml, calculated upon the volume of extract, and is considerably stronger than 1100 mμA, the original activity. This fact may indicate that an inhibitor is eliminated by the 50 % ammonium sulphate addition. The activity 1900 mμA should theoretically be the sum of the activities in Sup. 75 and Prec. 75, and it will be seen that these values are together about 2000 mμA/ml, which is a fairly good agreement.

Table 23. Fractionation of an extract with ammonium sulphate.

Ammonium sulphate was added to 50 % saturation at pH 4.5 to a part of the extract studied in Table 13. The mixture was kept 8 hours in the cold and then centrifuged (5000 × g). The resultant precipitate rose to the surface, it was called Prec. 50 and was dissolved in distilled water to the original volume. The clear solution was called Sup. 50. 1.00 ml solution to be measured was mixed with 3.00 ml gelatin and measured at pH 4.3.

Exp. No.	Preparation	$\frac{m\mu A^{I}}{ml}$	$\frac{m\mu A^{II}}{ml}$	$\frac{m\mu A^{II}}{ml}$ orig. vol.
88	Sup. 50	0	560	1 120
89	Prec. 50	55	1 090	1 090

Comparison of the values in Table 23 with the activity of the fresh extract in experiment 49, Table 13, which was 896 mμA/ml shows that both in the precipitate and in the clear solution the activity is higher and together more then twice the former activity. This is probably due to an inhibitor being split off by the ammonium sulphate treatment. This is also in agreement with the results in Table 22. A known example of this splitting off of an inhibitor is the case of the plasmin-inhibitor complex when fractionated with ammonium sulphate (cf. (17)).

In conclusion it may be said that the results in Tables 22 and 23 indicate that the enzymatic activity is increased by fractionation with ammonium sulphate, which may split off some inhibiting factor. Attempts to isolate this inhibitor have so far been unsuccessful.

Some experiments on the stability of the catheptic enzyme

The enzyme present in acid extracts and homogenates was subjected to some investigations regarding heat stability, stability at different pH and determination of the rate of decomposition. The results are reported in the text, Table 24 and 25, and in Fig. 4.

The extract ARS (a frozen and thawed aqueous extract from sediments obtained by extraction with water of fresh jelly coat-free *Arbacia* eggs with subsequent acidification and centrifugation), studied in Table 10 was kept at 58° C (pH 4.4) for different intervals of time (0–60 minutes) and the activity was measured at pH 4.3. The original activity without the addition of cysteine was 590 mμA/ml and decreased rapidly with time, being only 20 mμA/ml after 60 minutes. When, however, cysteine was added, the activity could be restored to a high degree, even after heat incubation, being 850 mμA/ml in the untreated extract and 580 mμA/ml after 60 minutes' incubation. Thus incubation for 1 hour at 58° C caused a decrease of only about 30 %. A further study of this extract was then made. Samples were incubated for 30 minutes at different temperatures (50° C–67° C). As could be expected a decrease in activity was found at 50° C and 55° C, but at 58° C and 60°C an increase occurred, followed by a decrease at 65° and 67°. So far there has been no opportunity to repeat this investigation on fresh *Arbacia* extract.

A similar investigation upon an extract from lyophilized *Paracentrotus* eggs is shown in Table 24. The pH-stability-curve of an *Arbacia* homogenate is shown in

Fig. 4, and the stability of the catheptic enzyme in an extract of lyophilized *Paracentrotus* eggs studied at an elevated temperature (60°) is shown in Table 25.

Table 24. Termostability of the catheptic enzyme.

Unfertilized, lyophilized *Paracentrotus* eggs were extracted in citrate buffer (4.4), frozen, thawed, kept overnight at + 3° C and centrifuged. 1.24 mgN/ml. Samples were kept for 30 minutes at different temperatures and 1.00 ml was mixed with 3.00 ml gelatin and measured. Cysteine was added to each sample after the heat incubation but before the measurement. pH 4.4.

Exp. No.	Temperature at incubation	mμA/ml
90	—	940
91	50°C	490
92	55°C	115
93	58°C	95
94	60°C	50
95	65°C	25
96	70°C	0

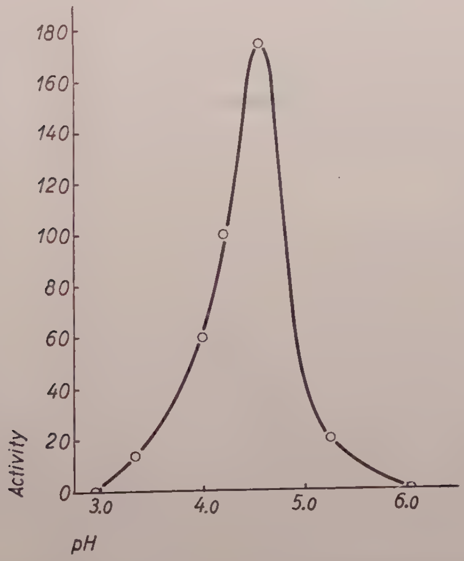

Fig. 4. Stability-pH-curve of *Arbacia* catheptic enzyme.

Unfertilized *Arbacia* eggs were washed twice in sea water and twice in isotonic NaCl, homogenized in citrate buffer pH 4.3 and centrifuged. 1.60 mg N/ml. Cysteine (0.01 M) was added to the homogenate. Each sample was heated for 30 minutes at + 60°C at the different pH values determined (HCl or KOH). After the incubation period the pH was changed to the original value of pH 4.3 and the activity measured after mixing 1.00 ml of the sample with 3.00 ml gelatin. Two measurements at each pH were performed. The original activity of the cysteine activated homogenate was 600 mμA/ml. The activity in the figure is expressed as mμA/ml.

The activity values in Table 24 show a continued decrease in activity with increasing temperature. There is no similarity with the unusual results of the *Arbacia* extract ARS (se above).

The stability-pH-curve of the catheptic enzyme from citrate homogenized *Arbacia* eggs is shown in Fig. 4. The optimal stability appears to be about pH 4.5, which is near the optimum for activity (cf. Fig. 1). The range of pH for stability is rather narrow. EDER et al. (18) report that liver cathepsin has maximum stability from pH 3.5 to 5 which is in agreement with the present curve.

Table 25. Determination of the rate of decomposition of dialysed and non dialysed *Arbacia* homogenate.

Unfertilized *Arbacia* eggs, washed in sea water and isotonic sodium chloride in the ordinary way, were homogenized in citrate buffer (pH 4.3) and centrifuged. 1.60 mgN/ml. This homogenate is called preparation I. To a part of this, cysteine was added and the homogenate was dialysed against distilled water for 24 hours in the cold, and centrifuged. 1.17 mgN/ml. The dialysed homogenate is called preparation I Dial. After adjustment of pH to 4.3 (citrate buffer) and addition of cysteine the preparations were kept at 60° C and samples were taken at different intervals of time, mixed with gelatin, and mesaured.

Exp. No.	Preparation	Incubation time in min.	mμA/ml	mμA/mg N
97	I	0	975	610
98	I	5	600	375
99	I	10	510	320
100	I	20	375	235
101	I	30	200	125
102	I	45	105	65
103	I Dial.	0	64.5	55.1
104	»	5	60.0	51.3
105	»	20	33.0	28.2
106	»	50	15.0	12.8

In order to determine the time for the disappearance of 50 % (half life period) of the activity, the values were plotted on a logarithmic scale against time on a linear scale. When determined in this way the period of half life was found to be about 14 minutes for the strong undialysed homogenate, and about 24 minutes for the weak dialysed extract. After the dialysis less than 7 % of the basic activity is left, but the enzyme is more resistant to heat incubation as seen from Table 25. Possibly an instabilizing agent is removed by the dialysis. The results of Table 7 seem to support this idea. The decomposition appears to occur as a reaction of first order.

Benzoylarginine amide splitting activity

Some attributes of the enzyme in acid extracts or homogenates studied in the last section make it natural to classify the enzyme as a cathepsine. The activity optimum of the enzyme at about pH 4.3 and the activating influence of cysteine are common qualities for some of these enzymes. In 1939 FRUTON and BERGMAN (19) used 1-benzoylarginine amide as a substrate for a proteolytic enzyme of beef spleen activated by cysteine, which enzyme was designated (20) by FRUTON, IRWING and

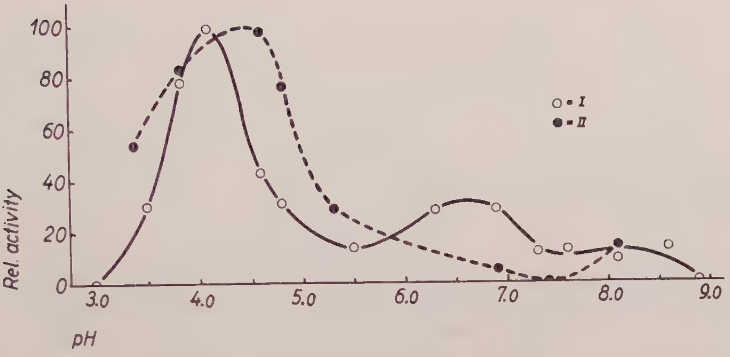

Fig. 5. Activity-pH-curves of extracts from *Paracentrotus* eggs.

Curve I: Lyophilized unfertilized *Paracentrotus* eggs were extracted in distilled water for 5 hours at +3°C and then centrifuged. 2.16 mg N/ml. The extract stored at +3°C was 1 week old at measurement.

Curve II: The same kind of material was extracted in citrate buffer, pH 4.1 at +3°C for 5 hours with subsequent centrifugation. 1.34 mg N/ml. The extract was 1 day old at measurement. The Conway technique was used. Substrate: 1-benzoylarginine amide (BAA).

Reaction mixture: 0.20 ml BAA + 1.00 ml buffer (citrate, acetate or imidazole) + 1.00 ml cysteine activated extract. 1.00 ml immediately after mixing and 1.00 ml after 21 hours digestion at 35.5°C was taken from each sample.

BERGMANN as cathepsin II. This type of enzyme has also been studied by GUSTAFSON (21) working with advanced stages of fertilized egg from different sea urchin species. In this work a homogenate possessing strong gelatin splitting activity was chosen for an investigation of a possible 1-benzoylarginine amide splitting activity and was prepared as follows: Unfertilized *Arbacia* eggs, freed from their jelly coats and washed in sea water and isotonic sodium chloride, were homogenized in citrate buffer at pH 4.3 and centrifuged.

The activity was first measured using gelatin as substrate as described below. The cysteine treated homogenate had an activity of 1580 mμA/ml. Following the addition of sodium periodate (0.005 M) to the homogenate-gelatin mixture 15 minutes after the start, the activity immediately disappeared, but was partially restored to 340 mμA/ml (pH 4.3) when cysteine was added 30 minutes after the start.

In order to determine the benzoylarginine amide splitting activity the following experiments were performed. In the active sample 2.00 ml cysteine treated homogenate was mixed with 2.00 ml H_2O and in the blank 2.00 ml homogenate (no cysteine) was mixed with 2.00 ml $NaIO_4$ (0.01 M). The samples were kept for 15 minutes at +20°C. From each of these samples 2.00 ml were taken and 1.60 ml acetate buffer (pH 4.3) and 0.40 ml BAA were mixed with each (cf. under methods) and kept at +32°C. The activity was measured at the start, after 10 hours and after 24 hours, and determined by the Conway method. The activity expressed as the light absorption (E) at $\lambda = 4300$ Å, and with the controls as references was:

Time in hours	0	10	20
Extinction E_{4300}	0	0.664	1.150

These values indicate a very strong BAA-splitting activity, which was also found in other homogenates from Arbacia at pH 4.4.

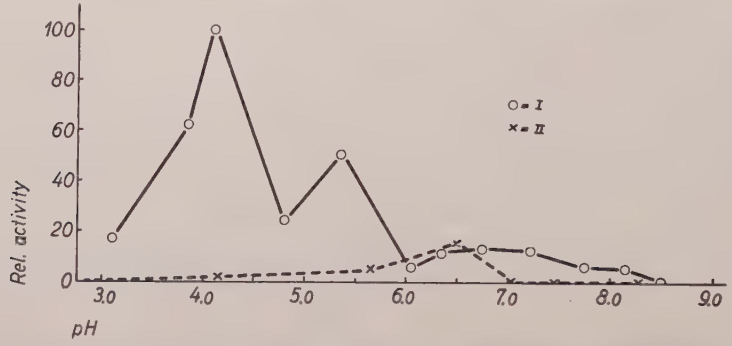

Fig. 6. Influence of cysteine on the BAA-splitting activity of aqueous extracts of *Paracentrotus* eggs.

Lyophilized, unfertilized *Paracentrotus* eggs were extracted in distilled water in the cold for 6 hours and centrifuged (5000 × g). Reaction mixture: 0.20 ml BAA + 1.00 ml buffer (as in Fig. 5) + 1.00 ml extract. Digestion: 21 hours at 35.5° C. Curve I: Cysteine treated extract. Curve II: No cysteine

The continued studies of the 1-bezoylarginine amide splitting activity made upon lyophilized *Paracentrotus* eggs and also on fresh extracts from *Brissopsis* oocytes are shown in Figs. 5–7.

The activity-pH-curves from one citrate buffered extract and one distilled water extract, both from unfertilized lyophilized *Paracentrotus* eggs are shown in Fig. 5. In the extract made in citrate buffer there appears on the curve a rather broad pH optimum around pH 4.5 with slowly decreasing activity towards pH 7. The aqueous extract curve has two pH-optima, one at pH 4.25 and another at about pH 6.6 rather flat and low. There is also some activity in the interval pH 7.5 to 8.75.

The influence of cysteine upon an aqueous *Paracentrotus* extract is shown in Fig. 6. The series, which was not cysteine activated, shows a very slowly increasing activity from pH 4, with an optimum at about pH 6.5 but without any activity at pH higher than 7. The cysteine activated extract has a strong optimum at pH 4.2, another at pH about 6.75 slowly decreasing towards pH 8.5. As seen in the figure there is also

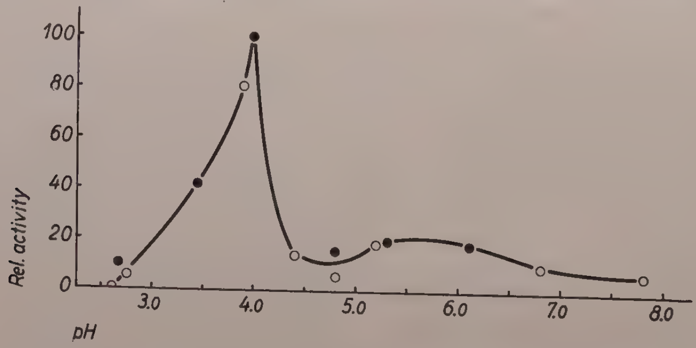

Fig. 7. Activity-pH-curve of extract from *Brissopsis* oocytes.

The *Brissopsis* extract (II) studied in Table 7 was investigated. Substrate: BAA. Reaction mixture: 1.00 ml extract II (cysteine act.) + 1.00 ml buffer + 0.20 ml BAA. 1.00 ml was taken from each sample immediately after the mixing and 1.00 ml was taken after 21 hours digestion at 35.5°C. Two series were run on the same extract (indicated by different symbols in the figure).

high activity at pH 5.3. If this is indeed the case, the varying results from extracts made in acetate buffer and reported below seem to be of interest.

A couple of activity-pH-curves were made on acetate buffered extracts (pH 4.5) of lyophilized *Paracentrotus* eggs. Some of these curves indicate two pH optima on the acid side, one at pH 3.8 and another at pH 5.25. Other series had, however one optimum at about pH 4.0–4.5. Altogether these results gave a rather confusing picture of an activity-pH-curve from eggs extracted in acetate buffer. GUSTAFSON (21) who found an optimum at pH 4 (*Psammechinus* gastrulae: hemoglobin) also reports (22) occasional activity at pH 5.5.

Finally the activity-pH-curve of an extract from *Brissopsis* oocyte is shown in Fig. 7. There is a sharp peak at pH 4 and a flat optimum at pH 5.5, slowly decreasing towards pH 8. It is interesting to note that at pH 6.6, there was no gelatin splitting activity in the original extract I (Table 7).

A preliminary investigation on the influence of carcinogenic hydrocarbons on the catheptic enzyme

Some experiments on the influence of two carcinogenic hydrocarbons upon the catheptic activity of citrate buffered extracts from unfertilized lyophilized *Paracentrotus* eggs have been carried out and the results will briefly be given here. The incubations were made by the method of RONDONI and BARBIERI (23). One ml of a 0.5 % solution of the carcinogen, dissolved in acetone, was suspended in 10 ml boiling distilled water, and 0.50 ml of this suspension was added to 1.00 ml *Paracentrotus* extract and the mixture kept for 30 minutes at + 20° C before mixing with gelatin. 20-Methylcholanthrene and 3,4-benzpyrene were tested. Methylcholanthrene had no effect upon the activity of the extract, whether cysteine was added before or after the incubation. 3,4-benzpyrene increased the activity of cysteine activated extract about 25–30 %. This effect is rather confusing as RONDONI and BARBIERI and other authors report decreased activity of cathepsin after treatment with some carcinogenic hydrocarbons. The assumption that an inhibitor is blocked by the hydrocarbon might explain the activation.

Discussion

The discovery that a transient activation of proteolytic enzyme of SH type occurs immediately after fertilization of the sea urchin egg, demonstrated by the writer (1, 2, 3), is supported by the observations of RUNNSTRÖM and KRISZAT (24, 25), working with eggs from sea urchins, which authors report a period of greatest susceptibility to chemical SH inhibitors, such as o-iodosobenzoic acid and p-chloromercuribenzoate, during the first 30 minutes after fertilization.

The most important problems involved in the present studies concern the occurrence of inhibitors in homogenates and extracts of eggs from some sea urchin species, the influence of different homogenization media upon the activity of the proteolytic enzymes in these homogenates, and the nature of the enzyme active at pH 4.3.

All investigations made on the enzymes active in a neutral medium show that this enzymic activity is irregular in occurrence and unstable. Experiments by GUSTAFSON (22) indicate the existence of a proteolytic enzyme component with maximum activity near the neutral point. The enzyme has irregular activity which seems to be regulated by conditions which it has so far been impossible to control (cf. (21)).

The degree of dilution might influence the proteolytic activity of a homogenate. JEFFREE (26) found this to be the case in preparations of prosthatic acid phosphatase which lost their activity on dilution in physiological saline, whereas full activity was maintained on dilution in serum. The "operating" factor appeared to be a relatively small organic molecule.

Only a few experiments concerning the influence of calcium have been made in the present work. It was reported earlier (cf. (3) Table 30) that calcium had no activating effect upon a dialysed extract, and it was found during the present investigations that calcium had no power to increase the activity in old extracts, which could be activated by cysteine at pH 6.5–6.7. However, it has now been reported by GROSS (27) that water soluble proteins in homogenates from *Arbacia punctulata*, when precipitated with ammonium sulphate, redissolved and dialysed showed proteolytic activity at pH 6.6 after the addition of calcium. It may be possible that in the course of the ammonium sulphate fractionation an inhibiting factor is split off, which otherwise destroys the enzyme. This is evidently the case in dialysed oocyte homogenates, which became more active after dialysis when cysteine was added (see Table 7). However the mechanism of activation by calcium and by cysteine is still very obscure. The effect of calcium on cathepsin II is both activating and stabilizing (cf. Table 21), a 0.01 M concentration of calcium in the incubation mixture being most favourable.

The experiments on reactivation of the catheptic activity in homogenates inhibited by sulphydryl reagents (Tables 16–18) indicate the presence of an inhibitor of SH type. Attention may be called to recent investigations by ROTH (28) of a liver ribonuclease inhibitor of sulphydryl type, which is blocked by SH reagents at neutral pH thereby increasing the activity of the enzyme in homogenates. Moreover results from ammonium sulphate frationation of extracts (cf. Tables 22 and 23) suggest the presence of an inhibiting factor which is split off by the salt (pH 4.3). The existence of an inhibitor operating at neutral pH could not be demonstrated by dialysis (Table 1), as the enzyme active at this pH is of a most unstable type. Whether the strange inhibiting effect of cysteine, which was reported in Tables 5 and 16 in a previous paper (3), and the activating influence of hydrogenperoxide shown in Table 8 (3) are due respectivly to activation and destruction of an inhibiting factor is not yet clear.

The occurrence of enzyme inhibitors systems in extracts from the sea urchin *Strongylocentrotus pulcherrimus* is clearly demonstrated by the investigations by ISHIDA (29). Ishida found that concentrated centrifuged blastula water, collected just as the fertilization membrane was dissolving, possessed the ability to dissolve the fertilization membrane, whereas distilled water extract of late free swimming blastula inhibited dissolution of the membrane.

The gelatin splitting activity of cathepsin has been denied by ANSON and MIRSKY (30) who maintain that this effect (increased by cysteine) is due to the presence of carboxypeptidase, which should split gelatin and which is activated by cysteine. However SMITH and HANSON (31) have later demonstrated that carboxypeptidase, though it has the SH groups essential for activity, is completely inhibited by cysteine, the enzyme being a metal protein. Experiments made in the present work with a view to classifying the enzyme which is active around pH 4.3, fully support the assumption that the enzyme is of cathepsin II type. Thus it splits 1-benzoylarginine amide in the presence of cysteine, whereas ascorbic acid has no influence. The low activity in glycerol extract differs however from the general experiences. GUSTAFSON's results (21) also show the presence of cathepsin II in developed sea urchin eggs.

As experiments have shown that the proteolytic activity of homogenates in vitro is not influenced by the presence of jelly coats, these were not removed in all preparations. Since the jelly coats protect the eggs during washing it was judged preferable not to remove the coats beforehand. When acting upon a papain solution a noticeable difference occurred, however, both jelly coat free homogenate (I) and that containing jelly coats (II) exerted a strong activating influence upon the papain solution, but in the mixture of homogenate and gelatin (I) the activity became twice as strong as in the gelatin-homogenate (II) mixture, and furthermore, the activity decreased rapidly in the latter mixture, approaching zero after a short time, whereas in the former the activity was normal. In all experiments the substrate was gelatin and the pH during the measurements 6.5. Thus the homogenate containing the jelly coats exerted a strong inhibiting effect on the papain subsequent to the transient activating influence.

The lacking influence of heparin upon the proteolytic enzymes in egg preparations at pH 4.3 and 6.7 found by the writer is in contradiction to the results of KLINGENBERG (32) who found that cathepsin from spleen was inhibited by heparin at pH 4.0 and 6.2.

SUMMARY

1. Proteolytic activity in homogenates or extracts of eggs from a couple of sea urchin species has been studied. The activity was in most cases determined viscosimetrically using gelatin as substrate. Formol titration was made in one series of determinations (vide infra). As substrate for determination of cathepsin II activity 1-benzyolarginine amide was used in several experiments and the activity determined by addition of Nessler's reagent and measurement in a spectrophotometer.

2. Activity-pH-measurements have been made on fresh cysteine activated homogenates of unfertilized *Arbacia* eggs, the proteolytic activity being determined by means of formol titration. Three optima were found, one around pH 4.0–4.4, one at pH 6.6–6.9, and one at pH 7.8.

Undialysed homogenate could be activated by calcium or cysteine (pH 4.3). After dialysis, which destroyed most of the activity, only about 3 per cent of the activity could be restored by cysteine, whereas calcium had no action (pH 6.5).

3. In some homogenates from unfertilized *Paracentrotus* eggs activity was found at pH 4.3, 6.5 and 7.8. The activity at pH 6.5 disappeared rapidly and could not be reactivated by cysteine.

4. Fresh extracts from blastula and early plutei stages of *Paracentrotus* showed increased activity at pH 6.5 compared with the extract from the unfertilized eggs. It was necessary to add cysteine to all the extracts.

5. The high proteolytic activity at pH 4.3 (cathepsin II) was not increased in fresh extracts form material taken 8 minutes after fertilization of *Paracentrotus* egg when compared with extracts from unfertilized eggs.

6. Extracts from unfertilized, and 3 stages of fertilized eggs (5, 10 and 40 min.) possessed an activating effect upon a papain solution which decreased with increasing time from fertilization.

7. Extracts from unfertilized *Echinocardium cordatum* eggs possessed a strong activity at pH 4.4.

8. Extracts of oocytes from *Brissopsis lyrifera* showed high activity at pH 4.4, which decreased by 90 per cent during dialysis, but which was reactivated by cysteine to an extent about 50 per cent higher than the original value.

9. Homogenization in 30 per cent glycerol gave much lower activity at pH 4.3 than analogous preparations in distilled water. Homogenization in 0.25 mol sucrose solution gave strong activity at pH 4.3.

10. Homogenization of unfertilized *Arbacia* eggs in isotonic sodium chloride and in buffered solutions with subsequent short time extractions in distilled water, followed by cryolytic extraction for a longer period, show that the enzyme active at pH 4.3 (cathepsin II) is bound to granular parts of the cytoplasm, though sometimes partly liberated when the egg cell is disintegrated.

11. Experiments on inhibition and reactivation of acid homogenates (pH 4.3) indicate the presence of an SH-dependent inhibitor, which can be reactivated to a lower degree by cysteine. It is also possible by means of ammonium sulphate fractionation of the enzyme to get increased activity which may be further evidence for the exeistence of an inhibitor.

12. According to generally accepted nomenclature the enzyme with optimal gelatinase activity at pH 4.3 is a cathepsin II, because it splits 1-benzyolarginine amide in presence of cysteine. It is found in *Arbacia*, *Paracentrotus*, *Echinocardium* and *Brissopsis*. Different buffered extracts of lyophilized *Paracentrotus* eggs give somewhat divergent pH optima being around pH 4.0–4.5. There is also some activity at pH 6.7 and around pH 8.

13. Calcium, when added in appropriate concentrations seems to exert both activating and stabilizing influence upon the catheptic enzyme.

14. The optimal stability of the catheptic enzyme is around pH 4.5. The half-life period of the catheptic enzymes is about 14 minutes at 60°C for an undialysed homogenate and 24 minutes for the dialysed homogenate.

15. 3,4-benzpyren has an activating effect upon cathepsin II from lyophilized *Paracentrotus* eggs.

16. Jelly coats seem to have no influence upon the proteolytic activity when present in homogenates, but the eggs are easily damaged when freed from the jelly coats, and the enzyme active near pH 6.6 may leak out into the washing medium.

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Starch degradation by the α-amylases

II. Paper chromatography of low-molecular degradation products

By Karl Myrbäck and Ebba Willstaedt

With 3 figures in the text

Possible end-products of the α-amylase action on starch (amylopectin, glycogen) are the following saccharides (the meaning of the schematical formulas used here has been explained in earlier papers, cf. (1)):

I. *Glucose* which may be formed from starch *etc.* in two ways:

1) secondarily from maltose through the action of maltase, and 2) as a primary product of α-amylase degradation of starch or dextrins. At least one α-amylase, *viz.* malt α-amylase, forms considerable amounts of glucose without having any action on maltose; cf. (2).

II. *Oligosaccharides of the maltose series, i.e.* such containing α-glucosidic linkages exclusively ("maltose linkages"). In the following these oligosaccharides will be designated as M-2, M-3, *etc.*

a) *Maltose*, M-2, the chief degradation product, formed by all normal amylases (formula 2 : 1 in fig. 1).

b) *Maltotriose*, M-3 (formula 3: 1 in fig. 1) which, in contradiction to the statements of Meyer *et al.* (3, 4), is probably not hydrolyzed by all α-amylases, possibly by none of them (2, 5). Concerning isolation and properties of maltotriose, cf. (6, 7).

All higher oligosaccharides of the maltose series (maltotetraose, M-4, *etc.*) are hydrolyzed by the α-amylases and do not appear among the end-products of starch degradation.

III. *Oligosaccharides of the isomaltose series.* In the following, saccharides containing one 1,6-α-glucosidic linkage ("isomaltose linkage") together with a certain number of maltose linkages, will be designated as belonging to the "isomaltose series". Obviously this series may comprise isomers of the same degree of polymerization (DP), differing in the relative positions of the isomaltose and maltose linkages. An oligosaccharide of the isomaltose series, containing *e.g.* four glucose units, will be called IM-4, *etc.*

a) *Isomaltose*, IM-2, 6-α-glucosido-D-glucose, has been identified as one of the so-called stable dextrins originating from amylopectin (8); formula 2 : 2 in fig. 1. According to Meyer *et al.* (3, 4), this sugar is the only true starch (or glycogen) limit dextrin. This assumption, however, is not correct, cf. (2). The sugar is formed, although in relatively small quantities by certain amylase preparations, but it does not seem excluded that contaminating enzymes are involved in the reaction. In any case, even if isomaltose is one of the end-products of starch degradation it is certainly not the only limit dextrin.

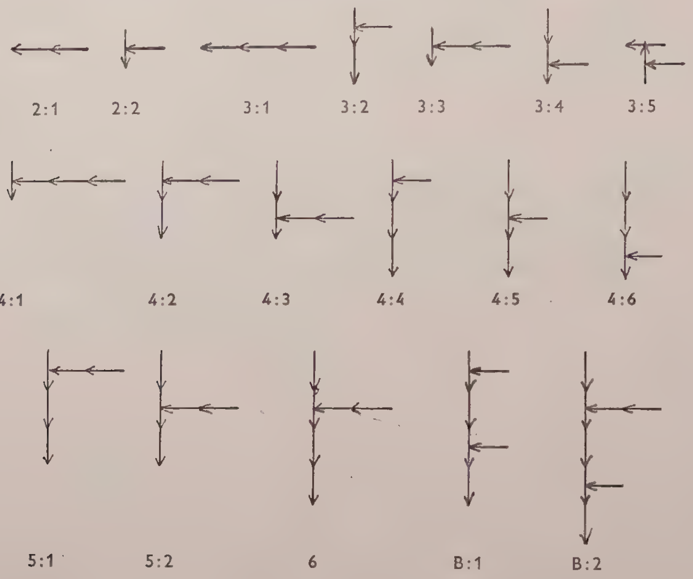

Fig. 1. Schematical representation of possible limit dextrins.

Isomaltose was isolated in the pure state by MONTGOMERY *et al.* (9) and by WOLFROM *et al.* (7).

b) *Trisaccharides*, IM-3, containing one maltose and one isomaltose linkage. Three isomers are possible: 1) 4-α-isomaltosyl-D-glucose, sometimes called *panose* (10); formula 3 : 2 in fig. 1, 2) 6-α-maltosyl-D-glucose; formula 3 : 3 in fig. 1, and 3) 4,6-diglucosyl-D-glucose; formula 3 : 4.

The formation of a saccharide of this type, probably panose, from starch by the action of an α-amylase was demonstrated by MYRBÄCK and AHLBORG (11), and subsequently the sugar was isolated in crystalline form by WOLFROM *et al.* (12, 10).

Whether the two other trisaccharides of the isomaltose series are formed from starch as limit dextrins is not known, but does not appear impossible.

c. *Tetrasaccharides*, IM-4. Six isomers are theoretically possible; formulas 4 : 1–4 : 6 in fig. 1. The two isomers 4 : 1 and 4 : 6 should not occur among the stable dextrins since maltose can be split off from the non-reducing end-chains by the amylases. The isomer 4 : 4 may possibly also be excluded since maltose may be split off from the reducing end-chain. Whether this reaction can be effected by the α-amylases or some of them is not known with certainty.

d) *Pentasaccharides*, IM-5. As pointed out in an earlier paper (1) such pentasaccharides as 5 : 1 and 5 : 2 in fig. 1 may well be represented among the stable dextrins.

e) *Higher saccharides* of the isomaltose series, IM-6 *etc.* It seems possible that certain of the 15 possible isomers, as for instance No. 6 in fig. 1, should be stable against the enzyme action. Most of the isomers, however, would be split with the formation of maltose and IM-4.

IV. *Oligosaccharides, containing two isomaltose linkages* together with a certain number of maltose linkages. Since amylopectin and glycogen, as far as we know, are quite irregularly ramified, it does not seem impossible that in the polysaccharides, and especially in glycogen, three branching points could be situated in so close vicinity that the α-amylases are not able to rupture the interior chains between them. The existence of stable dextrins of the type shown in formulas B : 1 and B : 2 in fig. 1 is probably not excluded, but the yield of such dextrins would certainly be low.

The simplest saccharide of this series would be "isomaltotriose"; formula 3 : 5 in fig. 1.

V. *Oligosaccharides with more than two isomaltose linkages* must be rare, especially if amylopectin (starch) is the substrate.

As an introduction to a chromatographical study of the low molecular degradation products of starch and glycogen we have tried to establish the behaviour in paper chromatography of certain of the oligosaccharides most probable to occur as end-products (stable dextrins). Simple sugars and some oligosaccharides have been thoroughly investigated earlier in a large number of publications (13). Hydrolysates of potato amylose by means of malt α-amylase have been used by JEANES, WISE and DIMLER (14). These hydrolysates were supposed to contain the homologous series of oligosaccharides with α-glucosidic 1,4-linkages (maltose series).)

Experimental procedure

Whatman filter paper No. 54 was cut perpendicularly to the machine direction in strips about 12 × 45 cm and used for descending chromatography in the ordinary way. The solvent, butanol-pyridine-water 6 : 4 : 3, was allowed to drip off during the runs. The solutions to be tested were applied as 2 mm spots on a line 9 cm from the upper end; 3–5 samples on each strip. Capillary pipettes were used. If necessary, several spots were applied, after drying, on top of each other.

The strips were suspended in the ordinary way from glass troughs in high glass cylinders containing a small amount of solvent on the bottom. The cylinders were covered by glass plates; a hole in these plates, covered by a smaller glass plate, allowed the introduction of solvent into the trough. This was done after 1 hour equilibration. The temperature was kept constant = 22°. Generally the chromatograms were run for 20–30 hours after which the strips were dried in an oven at 100° for 70–80 minutes.

The spots were developed according to TREVELYAN, PROCTER and HARRISSON (15): The strips were dipped in a solution of silver nitrate in acetone-water, dried in air at room temperature, sprayed with 4 percent NaOH in 92 percent alcohol and again dried at room temperature. After developement of the spots the strips were soaked in 4 N ammonia, rinsed in water, dipped in NaSH solution, rinsed in running water, and dried.

The distance of the spot centers from the starting line (generally about 30 cm for glucose) was measured. Glucose was used as a standard, and all other sugars were characterized by the R_G-value, *i.e.* the ratio of the distance travelled by the sugar to that travelled by glucose.

Sugars. Glucose, maltose and lactose were of best commercial quality. Maltotriose (used in some experiments in table 2) was a preparation isolated by ethanol fractionation of hydrolysis products of a normal α-dextrin. It contained maltose but was free from higher saccharides. Isomaltose was prepared by hydrolysis of a pure specimen of the acetate. We are indebted to Dr. G. NEUMÜLLER for a gift of this material. A chromatogram of a mixture of glucose, maltose and isomaltose is shown in fig. 2, No. 3.

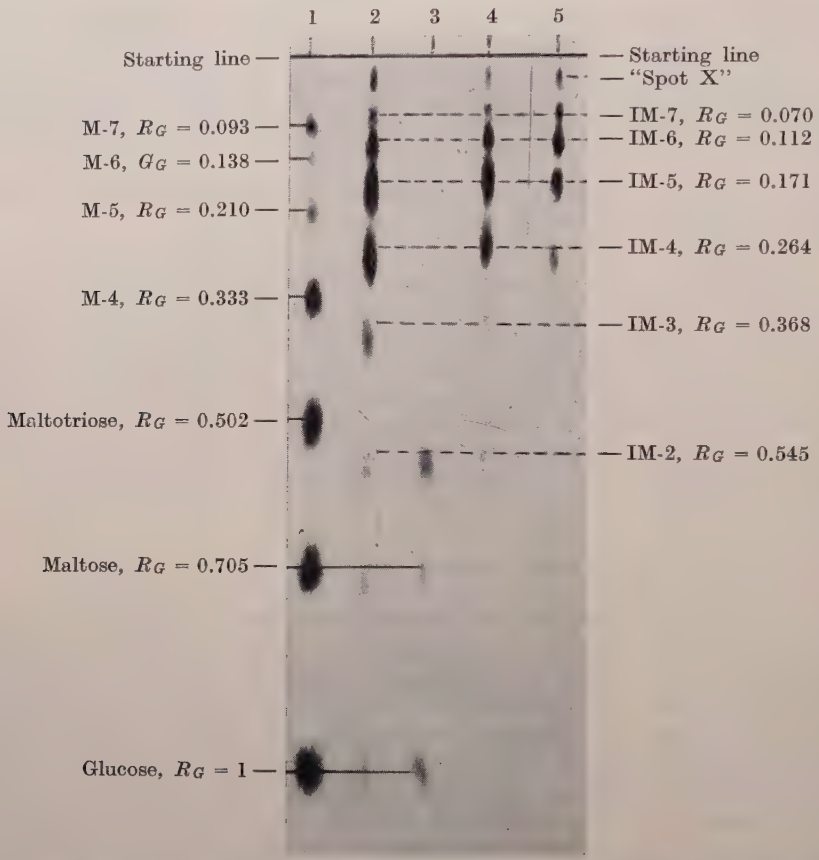

Fig. 2. No. 1: Sugars of the maltose series. No. 2: Hydrolysate of dextrin No. 3 (40 min.). No. 3: Glucose + maltose + isomaltose. No. 4: Hydrolysate of dextrin No. 3 (25 min., fermented). No. 5: Dextrin fraction No. 3.

Mixtures of glucose and the six first members of the maltose series were prepared by hydrolysis in *N* sulfuric acid at 100° of pure Schardinger β-dextrin (*Cyclo*heptaamylose). After 25 or 40 min., respectively, the hydrolysates were cooled, neutralized with pure $BaCO_3$ and filtered. Unchanged *Cyclo*amylose was removed by shaking the solution with tetrachloro ethylene, cooling and filtering. *Cyclo*amyloses in moderate amounts do not produce any spots on the chromatograms when developed as described above. The fact that seven spots were constantly observed on the chromatograms of the hydrolysates (No. 1 in fig. 2), *i.e.* that seven reducing hydrolysis products were present is, of course, a new proof of the correctness of the assumption that the Schardinger β-dextrin contains a seven-membered ring. (Hydrolysates of the same type as ours have been investigated by „circular" paper chromatography by Cramer (13), Tafel IV.)

Mixtures of saccharides of the isomaltose series were prepared by acid hydrolysis of a residual dextrin fraction, isolated in the following way:

15 l starch paste containing 870 g potato starch was treated for 19 days at 45° with 175 ml saliva. Apparent degree of hydrolysis approximately 50 percent. Fermentable sugars were removed by baker's yeast and the filtrate was reduced to a volyme of 1 litre. 800 ml were treated with

Table 1. R_G-values of the oligosaccharides belonging to the maltose series.

M-2	M-3	M-4	M-5	M-6	M-7
0.701	0.480	0.306	0.192	0.125	0.085
0.706	0.505	0.329	0.212	0.138	0.088
0.691	0.472	0.352	0.200	0.135	0.091
0.700	0.505	0.331	0.205	0.143	0.096
0.716	0.514	0.337	0.217	0.135	0.089
0.712	0.509	0.332	0.203	0.141	0.094
0.706	0.515	0.341	0.224	0.141	0.094
0.698	0.493	0.327	0.204	—	0.088
0.713	0.526	0.346	0.212	0.144	0.094
0.738	0.549	0.382	0.248	0.157	0.111
0.703	0.494	0.329	0.207	0.131	0.094
0.699	0.521	0.343	0.220	0.142	0.097
0.705	0.510	0.332	0.223	0.144	0.103
0.702	0.491	0.316	0.211	0.137	0.091
0.694	0.496	0.323	0.202	0.132	0.090
0.701	0.505	0.330	0.210	0.140	0.095
0.695	0.495	0.328	0.208	0.135	0.087
0.690	0.489	0.317	0.201	0.128	0.095
0.690	0.487	0.314	0.203	0.129	0.089
0.740	0.533	0.367	0.248	0.156	0.104
0.717	0.526	0.354	0.231	0.160	0.108
0.728	0.528	0.344	0.223	0.130	0.105
0.696	0.490	0.311	0.189	0.122	0.081
0.695	0.498	0.324	0.203	0.130	0.092
0.703	0.500	0.333	0.212	0.136	0.094
0.690	0.482	0.310	0.192	0.125	0.086
0.713	0.505	0.341	0.214	0.144	0.098
0.693	0.487	0.318	0.207	0.134	0.093
0.684	0.481	0.309	0.190	0.119	0.081
0.707	0.500	0.327	0.207	0.136	0.090
0.695	0.490	0.333	0.205	0.129	0.085
0.709	0.547	0.343	0.213	0.133	0.091
0.676	0.477	0.311	0.196	0.129	0.086
0.712	0.505	0.337	0.211	0.140	0.098
0.708	0.500	0.326	0.208	0.138	0.094
0.685	0.480	0.314	0.201	0.127	0.088
0.694	0.490	0.324	0.200	0.133	0.087
0.715	0.511	0.339	0.219	0.144	0.102
0.716	0.511	0.343	0.216	0.144	0.093
0.708	0.503	0.328	0.208	0.138	0.090
0.687	0.484	0.317	0.197	0.124	0.085
0.731	0.518	0.345	0.219	0.145	0.102
Mean values					
0.704	0.502	0.333	0.210	0.138	0.093

150 ml saliva for 24 days. No perceptible increase of the reducing power took place during the last week. After fermentation about 90 g of unfermentable dextrins was isolated. They were divided by alcohol precipitation into 7 fractions. Fraction No. 3 had an average degree of polymerisation = 6–7, and according to the paper chromatograms (No. 5 in fig. 2) it contained dextrins with 4, 5, 6 and seven units and, besides, a fraction containing at least 8 units ("spot X"). This

highest fraction may contain dextrins with two isomaltose linkages. To obtain a mixture of the lower members of the isomaltose series, as defined above, each containing one isomaltose linkage, samples of the dextrin fraction 3 were hydrolyzed in N sulphuric acid at 100° for 25 or 40 minutes, respectively. After cooling, neutralization with $BaCO_3$ and filtration, the hydrolysates were stored under toluene at 0°. Two chromatograms are shown in fig. 2, Nos. 2 and 4; No. 4 after fermentation with baker's yeast.

Results

Experiments, carried out during a considerable period of time, have been entered in the tables 1-5 in chronological order. Our aim was to test the reproducibility of the experiments, *i.e.* the possibility of characterizing certain oligosaccharides simply by their R_G-values. It is seen from the tables that generally the agreement is good, but there are a few single experiments in which the deviations from the mean values are not small. In most of these cases, however, it is seen that all values deviate in the same direction and by about the same relative amount. In these cases it can be said that the cause of the deviations is the value for glucose used in the calculation of R_G. Since glucose travels much faster than any other saccharide investigated here, it seems understandable that the position of the glucose spot in some experiments is less well defined than that of the other spots, especially as the glucose spot in some experiments was rather near to the lower end of the strips. Our experience is that the accuracy of the R_G-values decreases if the time of running is extended beyond about 24 hours. Besides, a R_G-value should be calculated from an experiment with glucose, added to the unknown sample, or at least from an experiment with glucose *on the same paper strip.*

Table 2. R_F-values of maltose and maltotriose.

Maltose	Maltotriose	Maltose	Maltotriose	Maltose	Maltotriose
0.700	0.488	0.700	0.482	0.692	—
0.700	0.506	0.703	0.514	0.709	—
0.677	0.432	0.677	0.464	0.700	—
0.721	0.514	0.712	0.453	0.696	—
0.720	0.520	0.711	0.496	0.694	—
0.704	0.500	0.695	0.489	0.693	—
0.693	0.496	0.701	0.477	0.688	—
0.702	0.517	0.710	0.512	0.744	0.554
0.693	0.496	0.714	—	0.740	—
0.678	0.464	0.720	—	0.731	—
0.685	0.467	0.733	0.536	0.724	—
0.686	0.472	0.735	0.540	0.734	—
0.712	0.516	0.731	0.542	0.729	—
0.710	0.504	0.712	0.508	0.750	—
0.674	0.449	0.704	—	0.726	—
0.668	—	0.712	—	0.736	—
0.654	0.444	0.693	0.498	0.711	0.506
0.699	0.492	0.685	0.489	0.722	0.512
0.707	0.485	—	—	0.716	—

Mean values: Maltose 0.706, Maltotriose 0.495.

Table 3. R_G-values of maltose and lactose.

Maltose	Lactose	Maltose	Lactose	Maltose	Lactose
0.716	0.546	0.711	0.544	0.714	0.538
0.711	0.535	0.736	0.559	0.708	0.538
0.713	0.560	0.743	0.566	0.706	0.532
0.716	0.533	0.723	0.559	0.716	0.538
0.718	0.553	0.711	0.542	0.702	0.525
0.742	0.551	0.721	0.550	0.709	0.528
0.720	0.551	0.689	0.522		
Mean values: Maltose 0.713, Lactose 0.544.					

Table 4. R_G-values of maltose and isomaltose.

Maltose	Isomaltose	Maltose	Isomaltose	Maltose	Isomaltose
0.667	0.496	0.712	0.547	0.705	0.550
0.712	0.553	0.695	0.541	0.745	0.594
0.690	0.530	0.691	0.539		
Mean values: Maltose 0.702, Isomaltose 0.544.					

Table 5. R_G-values of the saccharides of the isomaltose series.

IM-2	IM-3	IM-4	IM-5	IM-6	IM-7	?
0.533	0.360	0.260	0.170	0.111	0.069	0.025
0.530	0.357	0.250	0.154	0.100	0.065	0.019
0.545	0.361	0.262	0.167	0.114	0.073	0.024
0.553	0.380	0.272	0.181	0.117	0.073	0.024
0.533	0.366	0.259	0.168	0.108	0.063	0.023
0.543	0.367	0.260	0.169	0.109	0.063	0.025
0.564	0.375	0.271	0.176	0.117	0.075	0.029
0.548	0.375	0.270	0.175	0.118	0.076	0.020
0.554	0.367	0.273	0.176	0.119	0.075	0.032
Mean values						
0.545	0.368	0.264	0.171	0.112	0.070	0.025

When the determination of R_G is carried out as in our experiments, it does not seem possible to identify an oligosaccharide by the R_G-value from *one* experiment, but if a few are carried out and the mean of the R_G-value taken, it seems that the saccharides mentioned here may be well identified from them. For instance, the R_G-value of maltose is 0.705, that of isomaltose 0.545, and there should be no difficulty in distinguishing between them. (That the R_G-value of lactose happens to be 0.544 is of no importance in connection with starch degradation.) The R_G-value of maltotriose is 0.500 which is far enough from the isomaltose value to admit a clearcut distinction between the two sugars.

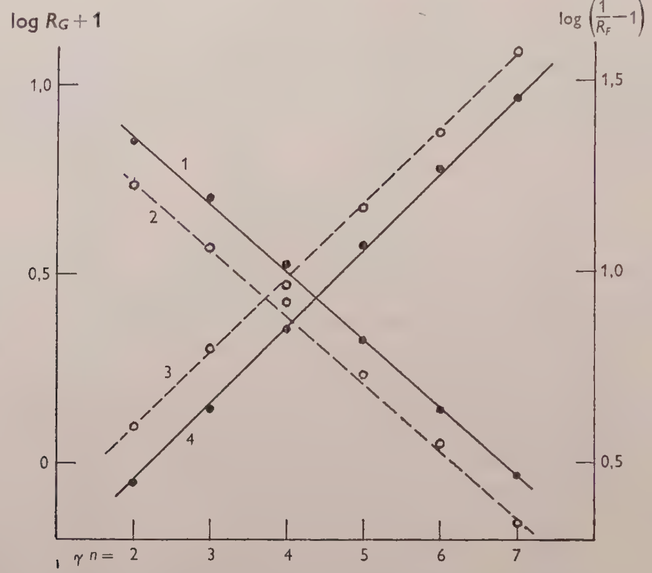

Fig. 3. Descending curves: log R_G plotted against n:
Curve 1, saccharides of the maltose series, Curve 2, saccharides of the isomaltose series.
Ascending curves: log $(1/R_F - 1)$ plotted against n:
Curve 3, saccharides of the isomaltose series, Curve 4, saccharides of the maltose series.

The two descending curves of fig. 3 show the connection between R_G and n, *i.e.* the number of glucose units in the oligosaccharides for the maltose and the isomaltose series. When log R_G is plotted against n, approximately straight lines are obtained. The same relationship was found by JEANES, WISE and DIMLER (15). (A linear relationship between log $(1/R_F - 1)$ and n has been found in certain homologous series (16, 17)).

Even if the two lines in fig. 3 are not absolutely straight they are obviously conform and well separated from each other. We have also calculated log $(1/R_F - 1)$, using the value $R_F = 0.37$ for glucose, found by JEANES *et al.* (15). When log $(1/R_F - 1)$ is plotted against n the two ascending lines in fig. 3 result.

It must be added here that whereas the saccharides of the maltose series (maltotriose etc.) are homogeneous it seems possible that the fractions which have been called here "oligosaccharides of the isomaltose series" contain more than one of the isomers mentioned. Therefore, we must conclude either that only one isomer of each degree of polymerization was present in our preparations or that such isomers can not be clearly separated by the method employed in our experiments.

SUMMARY

Saccharides, likely to occur as end-products of the α-amylase degradation of starch or glycogen, have been separated by paper chromatography and characterized by their R_G-values, *i.e.* the ratio of their velocities of migration to that of glucose. A separation of saccharides of the maltose and the isomaltose series up to a DP of at least 8 has been found possible.

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On the occurrence of vitamin B_{12}-factors in marine algae

By L.-E. ERICSON and LORAINE LEWIS

With 7 figures in the text

The presence in some marine algae of a growth factor for *Lactobacillus lactis* Dorner (ATCC 8000) and *Lactobacillus leichmannii* 313 (ATCC 7830) possessing the properties of vitamin B_{12b} has been reported from this laboratory [1, 2]. The concentration of this vitamin B_{12}-factor in the algae was found to be surprisingly high as compared with that of other plants, a few of which are believed to contain small amounts of the vitamin [3, 4, 5]. This has stimulated further research as to the distribution and origin of vitamin B_{12} in marine algae.

Several new species of algae have therefore been assayed for vitamin B_{12}. Bacteria living in the seawater and on seaweeds have also been isolated and their ability to produce B_{12} has been investigated.

A vitamin B_{12}-factor having features different from those reported for other members of the vitamin B_{12} group of factors has been found and will be referred to as vitamin B_{12s} in this paper.

Algae

The algae investigated are put together in Table I, which also describes their origin. Some algae that have already been assayed for vitamin B_{12} using lactobacilli and the results obtained mentioned in earlier papers [1, 2] have been reinvestigated and included in Table I. These algae are *Sphacelaria arctica, Laminaria saccharina, Furcellaria fastigiata* and *Rhodomela subfusa.*

Experimental. Several extraction procedures for the vitamin B_{12} were tried. Autoclaving or boiling dried and ground algae in water, with or without cyanide, is not suitable because of the simultaneous extraction of slimy material — e.g. alginates from the brown, agar-agar from the red seaweeds — that makes further purification and separation difficult. Attempts to prevent the alginic acid from dissolving by addition of an excess of $CaCl_2$ to the solution to form insoluble Ca-alginate were unsuccessful. Difficulties were also experienced in getting a complete extraction of the vitamin. Repeated freezing and thawing before extraction caused little or no increase in the amount of vitamin B_{12} released. Enzymatic digestion was without effect.

The best results were obtained when dried and finely ground material was boiled under reflux for about 30 minutes in a mixture of phosphate buffer and acetone (2 : 3) containing 0.04 % potassium cyanide (pH = 6.8). 50–100 ml of this mixture was generally used per gram dried weed. In spite of the large volume of solvent used, the process had to be repeated at least once before the extraction was complete. The combined extracts were evaporated to a final volume of 15–75 ml, depending on the concentration of vitamin B_{12} as judged from a preliminary test.

Table I. Vitamin B_{12}-factors present in twenty five marine algae. Bioautography with *E. coli.* The – signs mean that no investigation was made the 0 signs, that no activity was found. [1] = *Himanthalia lorea,* [2] = *Laminaria Cloustonii,* [3] from mud bottom (gyttja), [4] μg/ml see text. NS = North Sea, BS = Baltic Sea, ÅS = Åland Sea, AO = Atlantic Ocean. D indicates that due to high amounts of desoxyribosides no B_{12}-estimation could be made.

	Division		Order	Species	Total B_{12}-activity μg/gram		B_{12}-factors			Place and time f collection
					E. coli	*LLD*	B_{12}	ψB_{12}	B_{12s}	
Algae	*Rhodo(phyco)phyta*	*Florideae*	*Ceramiales*	*Laurencia pinnatifida*	0.27	D	+ + +	+	0	St. Andrews, NS
				Rhodomela subfusca	0.63	0.78	+ + +	+ +	+ +	Nämdö, BS
				Polysiphonia Brodiaei	0.20	–	–	–	–	North Berwick, NS
				Ceramium tenuicorne	0.50	D	+ + +	+	0	Nämdö, BS
				Ceramium rubrum	0.46	D	+ + +	0	0	North Berwick, NS
			Rhodymeniales	*Rhodymenia palmata*	0.09	0.07	+ + +	+	+	North Berwick, NS
			Gigartinales	*Chondrus crispus*	0.04	0.06	+ + +	0	0	North Berwick, NS
				Gigartina stellata	0.11	D	+ + +	+ +	+ +	North Berwick, NS
				Furcellaria fastigiata	–	0.05	+ + +	0	0	Simpnäs, ÅS
		Bangioideae	*Bangiales*	*Porphyra*	0.13	0.11	+ + +	0	0	North Berwick, NS
	Phaeo(phyco)phyta		*Fucales*	*Himanthalia elongata*[1]						North Berwick, NS
				fertile tracts	0.12	0.05	+ + +	0	0	
				vegetative thallus	0.06	0.004	+ + +	+	0	
				Pelvetia canaliculata						North Berwick, NS
				fertile tracts	0.075	0.08	+ + +	+ +	+ +	
				vegetative thallus	0.06	0.09	+ + +	+	+ +	
				Fucus vesiculosus						North Berwick, NS
				fertile tracts	0.04	0.05	+ + +	+	0	
				vegetative thallus	<0.004	0	–	–	–	
				Fucus serratus	<0.004	0	+ + +	0	0	North Berwick, NS
				Ascophyllum nodosum	<0.004	0	–	–	–	North Berwick, NS
			Laminariales	*Alaria esculenta*	0.12	0.14	+ + +	0	0	Lofoten, AO
				Laminaria hyperborea[2]						Dunbar, NS
				frond	0.06	0.065	+ + +	+ + +	0	
				stipe	0.10	D	+ + +	+ +	0	
				holdfast	0.26	0.26	+ + +	+ + +	0	
				Laminaria digitata	0.10	0.07	+ + +	+	0	North Berwick, NS
				Laminaria saccharina	0.01	–	+ + +	0	0	Fiskebäckskil, NS
			Sphacelariales	*Sphacelaria arctica*	0.10	0.12	+ + +	0	0	Simpnäs, BS

Table 1. (cont.).

Division	Order		Species	Total B_{12}-activity µg/gram		B_{12}-factors			Place and time for collection
				E. coli	*LLD*	B_{12}	ψB_{12}	B_{12s}	
Chloro(phyco)phyta	*Charales*		*Chara tomentosa*	0.21	D	+ + +	0	0	Öregrund^3, ÅS 10/52
		Siphonales	*Valonia ventricosa*						Virgin Islands, AO 5/53
			cell content	$<0.001^4$	–	–	–	–	
			cell wall	0.20	–	+ + +	0	0	
		Ulotrichales	*Ulva lactuca*	0.60	0.55	+ + +	+ +	0	North Berwick, NS 8/52
			Enteromorpha intestinalis	0.39	0.40	+ + +	0	0	North Berwick, NS 8/52
Chryso(phyco)phyta	*Heterokontae*	*Vaucheriales*	*Vaucheria dichotoma* . .	2.8	2.5	+ + +	+ +	+	Öregrund^3, ÅS 10/52

In the case of *Valonia ventricosa* the cell content and the cell wall of the plant were tested separately. The outside of the cell was washed with distilled water and sterilized with ethanol and formaldehyde. The cell wall was then punctured with a hypodermic and the cell content taken out. The cell sap — including the chloroplasts — was autoclaved (after addition of cyanide) and tested for vitamin B_{12}. The cell wall was washed several times in distilled water and autoclaved in 1.5 ml water containing small amounts of cyanide before determination of its vitamin B_{12} content.

The microbiological activity of the extracts was determined with the agar cup plate method using *Lb. lactis* Dorner (LLD, ATCC 8000) [6] and *E. coli* 113–3 [7, 8] as test organisms. For separation of the different B_{12}-factors both chromatography and ionophoresis on paper were employed. The solvent system for the chromatographic separation consisted of sec. butanol saturated with water and containing 3 % acetic acid and about 2.5 mg KCN per 100 ml. For the ionophoresis experiments phosphate, acetate and borate buffers were tried.

Results. The concentration and distribution of vitamin B_{12}-factors in the algae investigated is shown in Table I. The figures obtained for the total B_{12}-activity with the two microorganisms used agree fairly well considering the difficulty of obtaining reliable results with lactobacilli in the presence of desoxyribosides. Furthermore, different B_{12}-factors are generally differently active towards *E. coli* and *Lb. lactis* Dorner. In some cases the evaluation of an inner sharp, alkali labile growth zone with *Lb. lactis* Dorner became impossible due to the interference of desoxyribosides. This is designated by a D in the corresponding place of the table.

Figure 1 shows the position of the growth zones after chromatographic (Fig. 1a) and ionophoretic (Fig. 1b) separation. It is evident from Figure 1a that at least three different B_{12}-factors are present in most marine algae, i.e. vitamin B_{12},

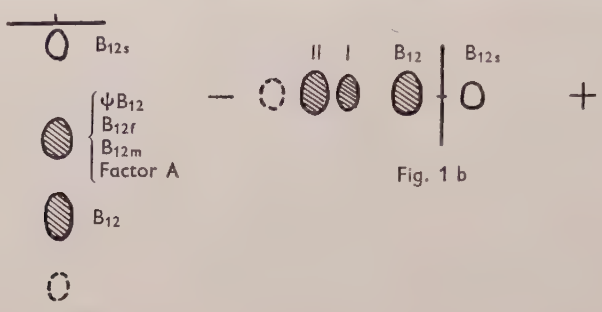

Fig. 1 a

Fig. 1 b

Fig. 1. The figure shows the chromatographic (fig. 1 a) and ionophoretic (fig. 1 b) behaviour of the vitamin B_{12}-factors found in the seaweeds and bacteria studied. Bioautography with *E. coli* was employed. I = main factor of pseudovitamin B_{12}, II = main component of factor A. The dotted spot indicates the presence of a factor — probably factor B — occasionally observed in extracts of some marine algae. Pseudovitamin B_{12}, vitamin B_{12f}, vitamin B_{12m} and factor A show almost identical *Rf*-values in the solvent system used for the chromatographic separation.

pseudovitamin B_{12}[1] and a third growth factor which has been given the name vitamin B_{12s}. Pseudovitamin B_{12} — as well as the B_{12}-factors mentioned in the footnote — gives only one spot on the chromatogram but separates into the two factors I and II (Figure 1 b) on ionophoresis on paper in an acetic acid buffer containing cyanide [9]. A growth factor for *E. coli* with a somewhat higher R_f-value than cyanocobalamin in the solvent system used and moving faster towards the cathode than the main component of factor A was occasionally observed in some algae, as indicated by the dotted spots in Figures 1 a and 1 b. It seems likely that this spot was due to factor B [9]. In Table I the number of + signs indicates the intensity of the growth zones as judged after chromatographic separation. It can be seen that most of the activity is generally caused by vitamin B_{12}.

About 100 grams of dried and ground *Rhodomela subfusca* were extracted twice, the combined extracts concentrated in vacuum and purified by adsorption on charcoal and chromatographed on an alumina column (twice). Ammonium sulfate was added to the combined eluates and the activity was driven into n-butanol. The butanol was shaken with distilled water and the pale yellow solution obtained was chromatographed on paper. The position of the spots of the vitamin B_{12}-factors in this purified extract was the same as in the crude extract of *Rhodomela*.

Figures 2 and 3 give the seasonal variation of vitamin B_{12} in *Alaria esculenta* and in *Laminaria hyperborea* (*L. Cloustonii*) over a period of about one year. The samples have been given to us through the courtesy of Mr. A. Haug, Norsk Institutt for Tang og Tareforskning, Trondheim, Norway, and are the same as were used by A. Haug and A. Jensen in their recent work on the seasonal variation in the main constituents of these algae [10]. Thanks to their kindness we have been permitted to plot into the same diagram their figures for the variations in proteins and ash, which allows a direct comparison between the seasonal changes in these constituents and vitamin B_{12}. Each figure in the diagram represents an average from more than 50 plants.

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[1] Pseudovitamin B_{12}, vitamin B_{12f}, vitamin B_{12m} and factor A appear to have almost identical *Rf*-values in the solvent used. The factor found in the algae having this *Rf*-value will be referred to as pseudovitamin B_{12}(ψB_{12}).

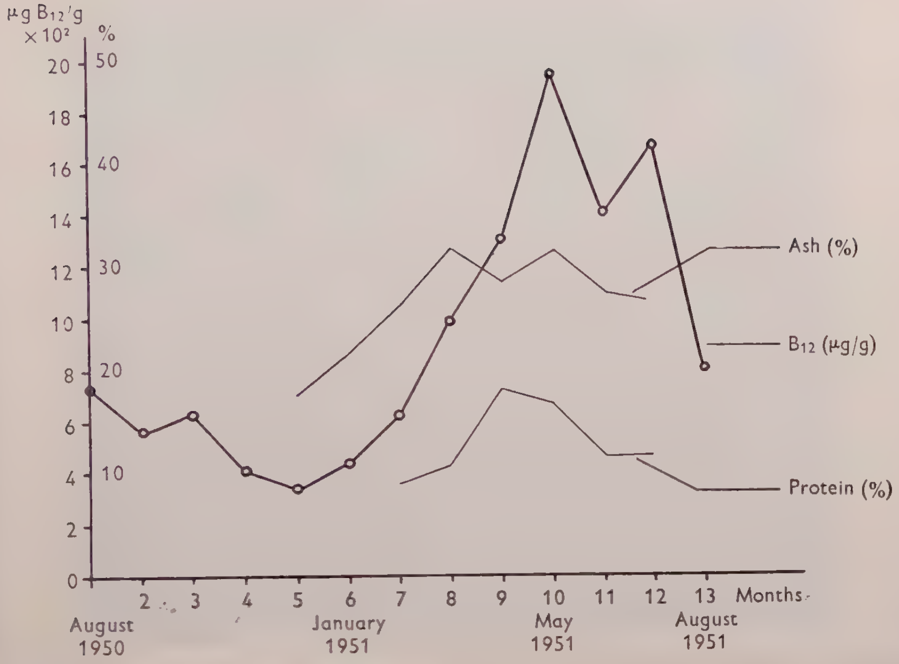

Fig. 2. Seasonal variation of the total vitamin B_{12} activity in *Alaria esculenta* from August 1950 to August 1951. The curves for protein and ash according to Haug and Jensen.

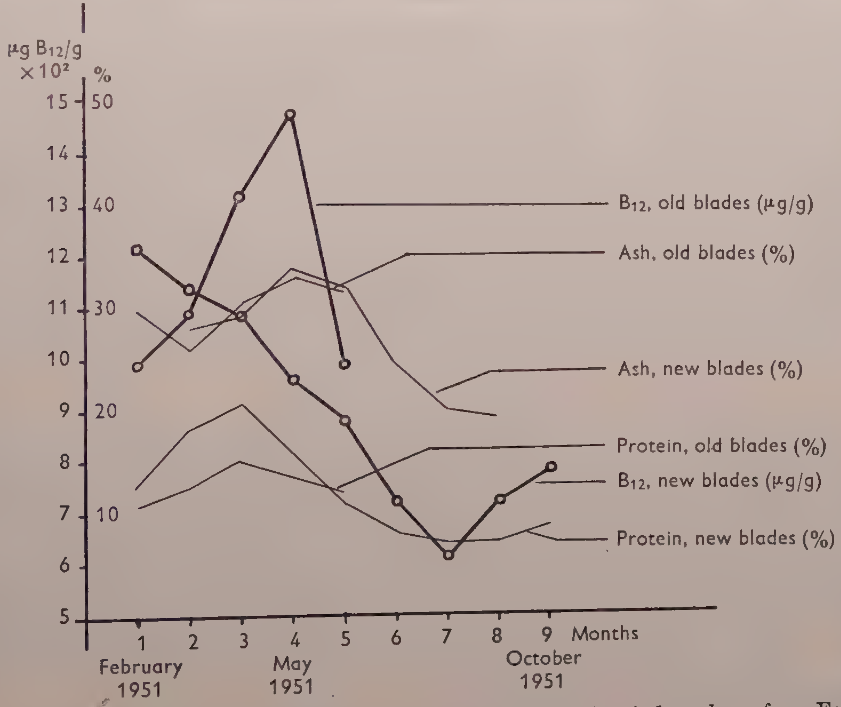

Fig. 3. Seasonal variation of the total vitamin B_{12} activity in *Laminaria hyperborea* from February 1951 to October 1951. The curves for protein and ash according to Haug and Jensen.

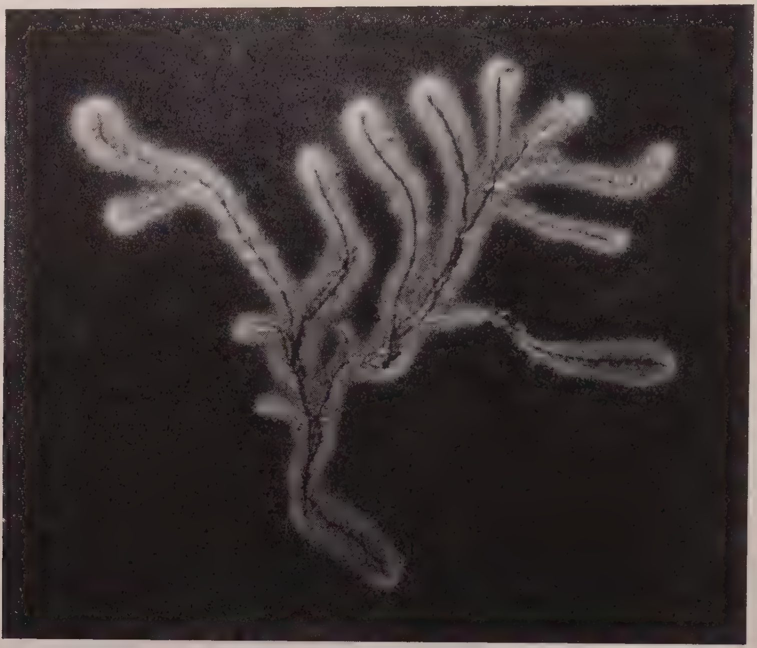

Fig. 4. Photograph of *Rhodomela subfusca* that has been placed on an agar plate seeded with *E. coli*. The white zone around the twigs is due to the growth of *E. coli* stimulated by vitamin B_{12} that diffused from the plant tissues into the agar.

Table I as well as Figure 4 gives an idea of the distribution of vitamin B_{12} within the algae. The fertile tracts of *Himanthalia elongata* and *Fucus vesiculosus* seem to contain more B_{12} than the rest of the alga. The result with *Pelvetia canaliculata* shows an almost equal distribution of vitamin B_{12} throughout the plant but may not be significant as this plant was harvested at a period when its fertile tips may have started to disintegrate. In *Laminaria hyperborea* the B_{12}-concentration at the time of collection was highest in the oldest part of the plant, i.e. the holdfast.

Figure 4 was obtained by placing a whole plant of *Rhodomela subfusca* on an agar plate seeded with *E. coli*. This was done after the alga had been washed in seawater, alcohol and distilled water and dried. The white zone around the black twigs of the plant is thus due to the growth of *E. coli* stimulated by the vitamin B_{12} diffusing from the plant tissues into the agar medium.

Microorganisms

The following section concerns itself with a study of some bacteria found living in the seawater and on three species of marine algae.

Experimental. The microorganisms were obtained from four different habitats: 1. Baltic seawater sampled October 1952 near Nämdö in the Archipelago of Stockholm; 2. *Rhodomela subfusca* taken at the same time and place; 3. *Fucus vesiculosus* gathered at Nämdö January, 1953; 4. *Vaucheria dichotoma* taken March, 1953 from Baltic brackish water at Öregrund (Åland Sea).

Microorganisms from sources 1 and 2 were first isolated on the following medium:

Bacto-Peptone	2.5 g	Ferrous ammonium sulfate	0.02 g
Yeast extract	0.5 »	Agar	7.0 »
Ammonium sulfate	0.17 »	Sterile seawater	500.0 ml

pH 7.4 after sterilization at 120° C for 20 minutes.

Microorganisms from sources 3 and 4 were first isolated on a medium containing:

Bacto-Peptone	2.5 g	Ferrous ammonium sulfate	0.02 g
Yeast extract	0.5 »	Magnesium chloride	0.6 »
Ammonium sulfate	0.2 »	Sodium sulfate	0.2 »
Sodium chloride	1.2 »	Calcium chloride	0.13 »
Agar	7.0 »	Distilled water	500.0 ml

pH 7.1 after sterlilization at 120° C for 20 minutes.

The seawater (1) was pipetted directly onto a sterile melted agar Petri dish in quantities ranging between 0.5 and 2.0 ml. Bacteria from seaweeds (2, 3, 4) were obtained in the following manner: small amounts of the plants were rinsed gently in sterile distilled water under aseptic conditions to remove organisms not actually attached to the plant. They were then put in 50 ml quantities of the above described liquid medium and incubated at 15–18° C for 4 days. Samples of the medium were then serially diluted with sterile distilled water and inoculated onto sterile melted agar in Petri dishes.

After 70 hours' incubation at 20–29° C, widely separated colonies of bacteria were inoculated onto agar slants. Pure cultures representative of the colonies present were obtained from the four samples. It is realized that some of the culturing conditions may have restricted growth of certain microorganisms.

All pure cultures obtained by these isolation methods were tested for vitamin B_{12} activity. 50 ml nutrient broth cultures were shaken in Ehrlenmayer flasks at 22° C for 72 hours. Samples, taken at 8–12 hour intervals were sterilized by autoclaving at 120° C for 20 minutes and the broth was tested for B_{12} by the cup plate assay using *E. coli* 113–3 [7, 8].

Cultures producing no vitamin B_{12} activity were discarded, the remaining were studied in respect of growth habits, chemical activities and morphology. All culture descriptions, chemical activity tests (including media, reagents and methods) were made according to the third edition of the Laboratory Manual on Fundamental Principles of Bacteriology by A. J. SALLE [11]. Morphological studies were made with a Reichert light microscope and a Phillips 100 kV electron microscope (type number 11980). A normal saline solution was used for dilution of organisms from an agar slant before applying to the collodion film of a specimen carrier.

Results. Of the 34 pure bacterial cultures obtained, 24 produced significant quantities of vitamin B_{12} and were studied further.

Table II gives characteristics of bacteria producing vitamin B_{12} in fermentation broth. Table III gives their probable classifications as determined with the aid of Bergey's Manual of Determinative Bacteriology [12]. Figures 5 and 6 show two electron micrographs of cultures 21 and 20 respectively.

This study compares favorably with results summarized by C. E. ZOBELL [13] for marine bacteria. The bacteria studied here were on the average smaller than those of soil and fresh water. The majority were motile rods and all were facultative aerobes. ZoBell recorded optimum temperatures ranging between 18 and 22° C for

Table II. Characteristics of twenty four cultures of bacteria isolated from seawater and from seaweeds. A = acid, Al = alkaline, G = gas, C = coagulated, R = reduced. Sugars used: G = glucose, L = lactose. [1] may be a mixture of two bacteria. + Activity, – no activity.

Cult. No.	Chemical processes													Morphology					Opt. Temp. °C	Facul. Aerobe
	Gelatin Hydrol.	Sugars		Starch Hydrol.	Casein Hydrol.	Nitrate Reduct.	H_2S	Indole	Methyl Red	Voges Prosk.	Urease	Catalase	Litmus Milk	Shape	Size	Spores	Motility	Color		
		G	L																	
1	–	A	–	–	–	+	–	?	–	+ +	–	–	(A)	Rods	1.65 × 0.70	–	–	–	25–28	+
2	+ +	A	–	–	–	+	–	+	–	–	–	+ +	AC	Rods	1.23 × 0.47	–	+	White	28	+
3	+ +	A	–	–	+	+	–	+ +	–	–	–	+ + +	AC	Rods	1.10 × 0.65	+	+	–	29	+
4	+ +	A	–	–	+ + +	+	–	–	–	–	–	+	AC	Rods	1.55 × 0.58	–	–	–	28	+
5	+	–	–	–	–	+	–	?	–	+ + +	+	–	A	Rods	1.06 × 0.53	–	+	–	27	+
6	+ + +	A	–	–	+	+	–	?	–	–	–	+ +	AC	Rods	1.45 × 0.64	–	+	White	27	+
7	+ +	A	–	–	–	+	–	–	–	–	–	+	A	Rods	1.10 × 0.44	–	+	White	27	+
8	+ +	A	–	–	–	+	–	+	–	+	+	+ +	AC	Rods	1.20 × 0.39	–	+	–	25–30	+
9	–	A	–	–	+	–	–	–	–	+	–	+ +	AC	Rods	0.85 × 0.54 2.40 × 0.32	–	+	–	30–32	+
10	–	A	–	–	+ +	–	+ + +	–	+	+	+	–	R	Rods	1.14 × 0.78	–	+	Orange	20–30	+
11[1]	+ +	A	–	–	+	+	–	–	–	–	–	–	AC	Rods	1.00 × 0.57 2.43 × 0.44	–	+	–	29	+
12	–	Al	–	–	–	+ +	–	+	–	–	+	+ +	Al	Rods	0.29 × 0.41	–	+	–	30	+
13	+ +	A	–	+ +	+ +	–	+ + +	–	?	+ + +	–	+	R	Rods	2.20 × 0.48	–	+	Orange	25–30	+
14	–	A	–	–	–	+	–	–	–	–	+	+ + +	Al	Rods	0.98 × 0.75	–	+	–	30	+
15	–	A	–	–	–	+	–	+	–	–	–	+	Al	Rods	1.75 × 0.38 6.80 × 0.49	–	+	–	30–32	+
16	–	A	–	–	–	–	–	?	–	+	+	+	–	Rods		–	+	–	25–28	+
17	–	A	–	–	–	–	–	+	–	+	+	–	–	Rods	1.07 × 0.48	–	+	–	30	+
18	+ + +	A	AG	+ + +	+ + +	+ + +	+	–	+	+ + +	–	–	ACG	Rods	1.44 × 0.72	–	+	–	20–30	+
19	+	Al	–	–	–	+ +	–	+	–	+ +	+	–	A	Rods	1.21 × 0.48	–	+	White	35	+
20	+ +	A	–	–	–	+	–	+	–	+ +	–	+ + + +	AC	Rods	1.10 × 0.21	+	+	Yellow	30–32	+
21	+ +	Al	Al	–	–	+	+	–	–	–	–	–	A	Rods	1.67 × 0.77	–	+	–	30	+
22	+	Al	Al	–	+	?	+	–	–	–	–	+	A	Rods	1.82 × 0.72	–	+	–	30	+
23	+ + +	A	Al	+	+	+	+	–	–	–	–	+	AC	Rods	0.97 × 0.49	–	+	–	25	+
24	+	Al	Al	–	–	?	+	–	–	–	–	+	A	Rods	1.81 × 0.79	–	+	–	30	+

Table III. Production of vitamin B_{12}-factors by bacteria isolated from seawater and seaweeds as well as their probable classification.

Cult. No.	Amt. of vit. B_{12} µg ml	Types of B_{12}-factors			Source of bacteria	Possible classification Class–*Schizomycetes* Order–*Eubacteriales*, Sub-order–*Eubacteriineae*	
		B_{12}	ψB_{12}	B_{12s}		Family	Genus
1	0.013	+ +	+ +	+	Seawater (1)	*Achromobacteriaceae*	*Achromobacter*
2	0.037	+	+ +	0	»	*Pseudomonadaceae*	*Pseudomonas*
3	0.12	+ +	+ + +	+	»	*Bacillaceae*	*Bacillus*
4	0.009	+ +	+ +	+	»	*Pseudomonadaceae*	*Pseudomonas*
5	0.007	+	+ +	0	»	*Pseudomonadaceae*	*Pseudomonas*
6	0.017	+	+ +	0	»	*Pseudomonadaceae*	*Pseudomonas*
7	0.009	+ +	+ + +	+	»	*Pseudomonadaceae*	*Pseudomonas*
8	0.010	+ +	+ + +	+	*Rhodomela s.* (2)	*Pseudomonadaceae*	*Pseudomonas*
9	0.020	+ +	+ + +	+	»	*Pseudomonadaceae*	*Pseudomonas*
10	0.005	+	+	0	»	*Achromobacteriaceae*	*Flavobacterium*
11	0.024	+	+	0	»	*Pseudomonadaceae*	*Pseudomonas*
12	<0.005	+ +	+ + +	+	*Fucus v.* (3)	*Achromobacteriaceae*	*Achromobacter*
13	<0.005	+ +	+ + +	+	»	*Pseudomonadaceae*	*Pseudomonas*
14	<0.005	+ +	+ + +	+	»	*Achromobacteriaceae*	*Flavobacterium*
15	<0.005	+	+ +	0	»	*Achromobacteriaceae*	*Achromobacter*
16	<0.005	+	+ +	0	»	*Achromobacteriaceae*	*Achromobacter*
17	<0.005	+	+ +	0	»	*Achromobacteriaceae*	*Achromobacter*
18	<0.005	+	+ +	0	»	*Enterobacteriaceae*	*Erwinia*
19	<0.005	+	+ +	0	»	*Pseudomonadaceae*	*Pseudomonas*
20	0.14	+	+ +	0	»	*Bacillaceae*	*Bacillus*
21	0.009	+	+	0	*Vaucheria d.* (4)	*Achromobacteriaceae*	*Achromobacter*
22	0.013	+	+	0	»	*Achromobacteriaceae*	*Achromobacter*
23	0.035	+ +	+ +	0	»	*Pseudomonadaceae*	*Pseudomonas*
24	0.015	+	+	0	»	*Achromobacteriaceae*	*Achromobacter*

bacteria from California coastal waters but he also reported Lundestad's work on bacteria from the Norwegian coast with optima ranging between 20 and 30° C. Our studies of bacteria from the Baltic Sea (brackish water, salinity about 0.55%) agree with the latter figures. We found fewer chromogens (only 5 of the 34 cultures) whereas ZoBell reports more than 50% as being chromogenic.

Little information can be obtained from the literature concerning bacteria actually living on marine algae. According to ZoBell, Reinke and also Keding observed fixation of nitrogen by *Azotobacter* growing epiphytically on algae [13]. ZoBell questions whether this is an example of true symbiosis between the bacteria and the plant. However, bacteria in the sea play an important role in the production of plant nutrients such as ammonia, nitrite, nitrate, sulfate, phosphate and carbon dioxide [13]. ZoBell also states that the secretions and excretions of living plants provide food for bacteria associated with them.

Table III summarizes the total *E. coli* activity of the bacterial cultures and also gives the types of the growth factors observed after bioautographic separation. The bacteria produced the same type of factors as those found in the algae. However, pseudovitamin B_{12} was produced in a higher concentration than vitamin B_{12} or vitamin B_{12s}. Vitamin B_{12s} seems to occur only during a certain stage of the culture experiments. An example of this is given in Figure 7 which shows the position and intensities of the growth zones obtained for samples taken at intervals

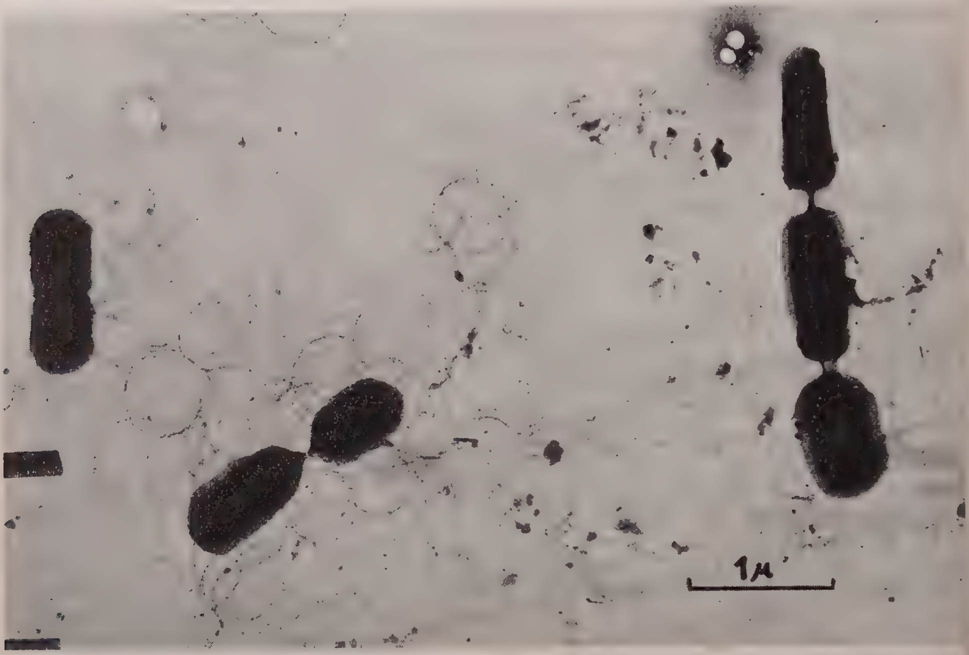

Fig. 5. Electron micrograph of bacterial culture 21, showing different stages of cell division and the presence of flagella.

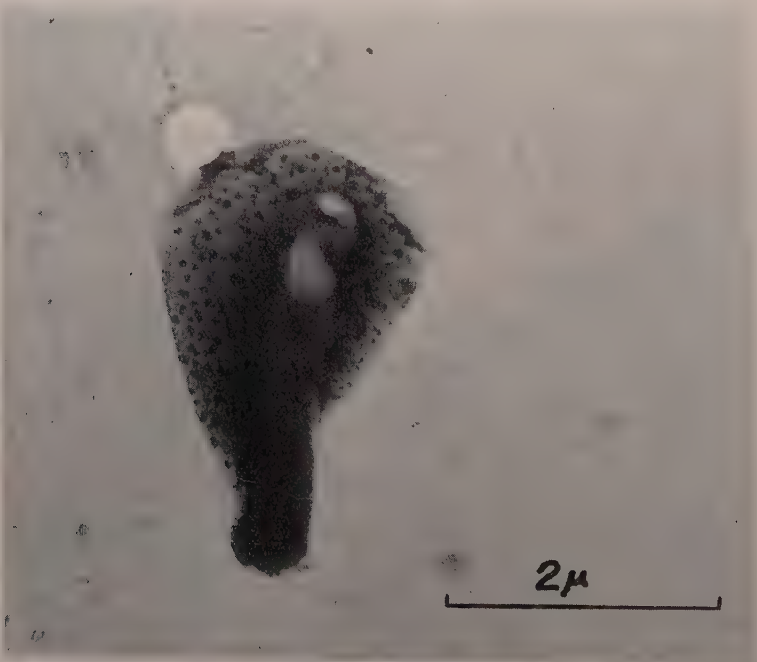

Fig. 6. Electron micrograph showing germinating spore form bacterial culture 20.

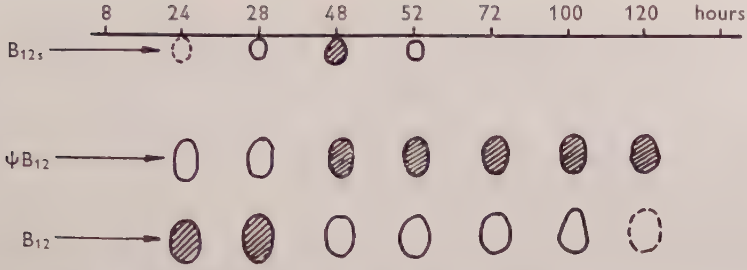

Fig. 7. Distribution of the vitamin B_{12}-factors in culture 3 fermentation broth at various stages of growth. The intensity of the spot due to vitamin B_{12s} showed a maximum after about 48 hours. At the end of the fermentation period pseudovitamin B_{12} was the main factor.

from a culture experiment with bacteria 3. The intensity of the spot due to vitamin B_{12s} reaches a maximum after about 48 hours. Addition of an excess of cyanide during extraction of the B_{12}-factors did not change this phenomenon. The total B_{12}-activity remained almost constant after 28 hours and was not influenced by the addition of cobalt (2 ppm).

The fermentation broth of culture 3 contained, during the first 48 hours of growth, a substance inhibiting the growth of *E. coli* and also of *Staphylococcus aureus*.

Four liters of fermentation broth from bacteria 3 containing vitamin B_{12s} was purified as described for *Rhodomela subfusca.* Paper chromatography showed that the position of the spot due to vitamin B_{12s} remained unchanged.

Discussion

Twenty-five different seaweeds have been assayed for vitamin B_{12}-activity. Only two of these did not contain B_{12}-factors in sufficient amount to be detected, i.e. they contained less than 0.004 μg/g dry weight. It thus seems that the presence of B_{12}-factors in marine algae is a very common feature for these plants. The concentration of vitamin B_{12} is low in the brown seaweeds (average for the examined species 0.07 μg/g), higher in the red algae (average 0.27 μg/g), and still higher in the green algae (average 0.35 μg/g). Outstanding features are shown by *Vaucheria dichotoma*, the only alga that belongs to the division *Chryso(phyco)phyta.* This alga contained not less than 2.8 μg B_{12}/g dry weight as estimated with *E. coli.* ROBBINS *et al.* [14] found B_{12}-contents in marine algae ranging from less than 0.0001 to 0.1 μg/g dry weight (*Euglena gracilis*). However, the samples of algae tested were four years old and the authors consider it probable that more complete methods of extraction would have given higher results. For further comparison it can be mentioned that guinea pig liver contains 0.69 μg B_{12}/g fresh tissue [15], egg yolk 0.48 μg/yolk [16], whole herring meal 1.3–1.5 μg/g dry weight [17], cows milk 0.004 μg/ml [18], fresh alfalfa 0.05–0.062 μg/g (more than 85% of this activity is believed to be due to factors other than vitamin B_{12}) [3], roots of beets and parsnips approximately 0.04 μg/g (rat assay, "Zoopherin") [5]. ROBBINS *et al.* report that roots of some higher plants contain 0.0002–0.01 μgB_{12}/g of fresh material as estimated with *Euglena gracilis* [4]. For whole clams PROCTOR and LANG have reported values of the order 6.0–9.7 μg/dry weight [19]. Shrimps contain from 0.0055 to 0.009 and oysters from 0.20 to 0.460 μgB_{12}/g fresh weight [14].

The isolated bacteria produced vitamin B_{12} in concentrations ranging from about 0.005–0.14 μg/ml fermentation broth. These figures are very low as compared to those obtained with some *Streptomycetes* (0.5–1.5 μg/ml), with *Flavobacterium devorans* (0.576 μg/ml), *Bacillus megatherium* (0.8 μg/ml) and some propionic acid bacteria (up to 4.3 μg/ml) [20]. A bacteria isolated from a pond where *Euglena* grew has been found to produce 0.01 μgB_{12}/ml [21], a figure which is more comparable to those obtained for bacteria isolated from seawater and seaweed.

The bacteria isolated from algae produced a greater vitamin B_{12}-activity when grown together than any of the strains by itself. A longer time of growth was required by such mixed cultures before maximum B_{12}-production was achieved.

It has been shown that marine algae are able to take up and concentrate radioactive vitamin B_{12} present in the seawater. The concentration factor varied from 20 to 120 times for different species [22]. The concentration of vitamin B_{12} in the sea must be extremely low. We failed to detect any vitamin B_{12}-activity in brackish water concentrated more than one thousand times. Attempts to absorb the vitamin in seawater on charcoal and to eluate it by boiling the coal in a mixture of water and acetone were also unsuccessful. From these experiments it is evident that the concentration of vitamin B_{12} in the seawater investigated must be well below 0.000005 μg/ml. Values of 0.0001–0.001 μg/ml have been reported for pools in which *Euglena* grew [4]. Two samples from a seashore (Nämdö, Stockholm archipelago) one mainly sand, the other decaying material, were found to contain 0.015 and 2.1 μgB_{12}/g dry weight respectively.

The amount of vitamin B_{12} found in marine algae can thus hardly be accounted for by direct accumulation from the seawater. Nor is it possible that the vitamin B_{12}-content of the algae could be due to the amount of vitamin B_{12} in the bacteria on the algae at the time of harvesting. The fact that bacteria living on seaweed have a definite, although comparatively poor, ability to produce vitamin B_{12} makes it probable that the vitamin B_{12} of marine algae has originally been produced by these microorganisms and subsequently taken up and stored in the plants. If one considers the ability of these algae to concentrate B_{12} from the surroundings one would get values that are of the same order of magnitude as those actually found. We have not been able to confirm the finding of other authors [14] that unicellular algae, when grown under strictly sterile conditions, are able to produce vitamin B_{12}. In those cases when vitamin B_{12}-activity was found it was also possible to detect bacteria in the algal cultures. It can also be mentioned that a sample of phytoplankton from Fiskebäckskil, North Sea, collected in the spring 1953 had no noticeable activity for *E. coli*, i.e. contained less than 0.001 μgB_{12}/g dry weight. Not only seaweeds but also submersed vascular plants can contain vitamin B_{12}. A sample of *Potamogeton pectinatus* was examined and found to contain 0.13 μg/g dry weight.

In collaboration with Dr. R. Scott of the Institute of Seaweed Research, Inveresk, Midlothian, Scotland, seaweeds have been grown in tanks with seawater containing radioactive Co^{60} and an attempt to isolate radioactive vitamin B_{12} has been made [23]. Antibiotics (streptomycin and chloromycetin) were added to the seawater in which the algae were grown to inhibit bacterial growth. Algae were also grown in water not containing antibiotics. The results of these investigations have not as yet given any indication that vitamin B_{12} should be produced by the alga itself, but rather suggest a microbiological origin.

Analysis of the cell content and the cell wall of *Valonia* showed that the content of vitamin B_{12} of this algae was due almost entirely to the vitamin B_{12} content of the cell wall. The cell content, that appeared to be sterile, contained less than 0.001 μgB_{12}/ml, whereas the cell wall had about 0.20 μg/g dry weight. The seawater in which the *Valonia* arrived contained 0.0025 μgB_{12}/ml and also bacteria. It therefore seemed as B_{12} from the seawater had been adsorbed on the cell wall of the *Valonia*, but that no or very little vitamin B_{12} had penetrated into the cell of the plant. The same types of vitamin B_{12}-factors were found in the water and the cell wall.

Assuming that the vitamin B_{12}-factors have not been produced by the alga itself, the question arises whether these vitamins are at all utilized by the seaweeds. When collected at the right time, fertile tracts seem to contain more B_{12} than the rest of the plant (Table I; see also Figure 4). However, as already mentioned, it was also noticed that in the case of *Laminaria hyperborea* the oldest part of the plant contained most vitamin B_{12}-activity.

The seasonal variation of the vitamin B_{12} of *Alaria esculenta* and *Laminaria hyperborea* shows that the B_{12}-content (whole plant) appears to have a maximum in May (Figure 2 and Figure 3). The ash content reaches a maximum at about the same time and the protein content approximately one month before the B_{12} maximum. None of the other plant constituents (alginic acid, laminarin and mannitol) bore any seasonal variation related to that of vitamin B_{12}. The total number of bacteria in the sea does not change considerably during the year and shows no maximum in April and May [13]. The increase of vitamin B_{12} in the spring can, in the case of *Alaria*, possibly be explained as caused by a higher general intake of nutrients during a period of more intense growth. The high B_{12}-content of the old blades of *Laminaria hyperborea* may be due to decomposition and growth of a greater number of bacteria on this part of the plant.

Red, green and blue-green unicellular algae have been grown under sterile conditions and vitamin B_{12} has been added to the cultures. None of the algae investigated by us seem to be significantly stimulated by the addition of vitamin B_{12} to the culture media, but the rather unexpected observation was made that several algae could convert cyanocobalamin into other B_{12}-factors, e.g. factors with the same R_f-values as pseudovitamin B_{12} and factor C. The algal flagellate *Euglena gracilis* is well known for its need of vitamin B_{12} [24]. *Chlamydomonas chlamydogama*, *Brachiomonas* sp. and *Lobomonas pyriformis* do also require vitamin B_{12}, as well as some chrysomonads [25, 26].

We have not been able to understand why the seaweeds consistently contain mainly vitamin B_{12} (Table I) whereas bacteria living on the weed produce mainly pseudovitamin B_{12} (Table III). It is not likely that the algae should be less able to accumulate pseudovitamin B_{12} than vitamin B_{12} considering only the structural differences between the two compounds. Experiments have shown that the higher content of vitamin B_{12} than of pseudovitamin B_{12} of the seaweed can hardly be explained as due to a higher stability of the vitamin B_{12} than of the pseudovitamin B_{12} at pH 8.0, which is the approximate pH of the seawater [27] (autoclaved at 120° C for 30 and 60 minutes). Vitamin B_{12s} which in bacterial culture is produced only during a certain period of growth and which appears to be more labile than vitamin B_{12} or pseudovitamin B_{12}, is present in many seaweeds. The bacteria isolated from *Vaucheria* did not produce vitamin B_{12s} although *Vaucheria* contains this vitamin B_{12}-factor. All B_{12}-factors seem to be firmly bound to some of the other constituents of the plant as it is difficult to release and extract them completely.

Summarizing these considerations of a possible metabolic role of vitamin B_{12}-factors in seaweeds it can be said that although some of the experimental data can be interpreted on the basis of a B_{12} requirement, none of these data nor the need of vitamin B_{12} by some unicellular algae seem to justify the conclusion that vitamin B_{12} is an indispensable growth factor for seaweeds. If there is any need at all for these B_{12}-factors, the amount required is probably much smaller than that found on analysis.

The amount of vitamin B_{12} found in any seaweed would probably be determined by the type of bacteria living on the weed and of the ability of the plant to concentrate the vitamin. It is interesting to note that bacteria living on algae containing a high amount of the vitamin generally showed a higher ability to produce it than those living on algae with a low concentration of vitamin B_{12}. Thus *Rhodomela subfusca*, containing about 0.7 $\mu g B_{12}/g$ dry weight was associated with bacteria that could produce as much as 0.015 $\mu g B_{12}/ml$ fermentation broth, whereas the bacteria associated with *Fucus vesiculosus*, which contains about 0.004–0.04 $\mu g/g$, produced less than 0.005 $\mu g B_{12}/ml$. The bacteria living on *Vaucheria* (2.8 $\mu g/g$) produced approximately 0.025 $\mu g/ml$.

It should also be mentioned that the same species of an algae seems to contain about the same amount of vitamin B_{12} when harvested at different places. *Fucus vesiculosus* from North Berwick (North Sea) and *Fucus vesiculosus* from Nämdö (Baltic Sea) thus contained about the same amount of vitamin B_{12}-factors. This has also been observed for *Polysiphonia* species, and for *Laminaria hyperborea.*

Vitamin B_{12s} has been compared directly with the following B_{12}-factors: vitamin B_{12} (cyanocobalamin, Merck & Co., Inc.), pseudovitamin B_{12} [28], vitamin B_{12f} [29], vitamin B_{12m} [30], factor A, factor B, and factor C[1] [31, 32]. It differs from these factors in its behaviour on chromatography and ionophoresis on paper. Vitamin B_{12s} cannot be identical with vitamin B_{12c} [33, 34] as this compound can be converted to vitamin B_{12} by addition of cyanide. Vitamin B_{12s} moves towards the anode at pH 7 as does factor C [9], which proves that vitamin B_{12s} contains negatively charged groups that are stronger than those of vitamin B_{12} (cyanocobalamin) [35]. The factor WR [30], that has recently been shown to be a mixture of several compounds [9], does not contain any component moving towards the anode. Vitamin B_{12s} is labile towards alkali and especially towards acids. It was not observed when the chromatograms were placed on plates seeded with *L. lactis* Dorner, and it must thus be assumed that it is much less active towards this organism than towards *E. coli.* A factor with chromatographic and ionophoretic properties similar to those of vitamin B_{12s} has been observed in a "vitamin B_{12b}" preparation from *Streptomyces aureofaciens* fermentation broth [36].

Occasionally a factor with chromatographic and ionophoretic properties like those of factor B [9] was noticed in some algae. Whether this factor actually occurs in the seaweed or if some of the other B_{12}-factors — probably vitamin B_{12s} — can be converted to factor B in a similar way as factor C_1 and factor C_2 [37] has not been further investigated. As no direct comparison between the factors C_1 and C_2 and vitamin B_{12s} has yet been possible, the possibility exists that C_1 or C_2 (probably C_2) and vitamin B_{12s} are identical.

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[1] A factor found in sewage sludge and showing the properties of factor C has been used for comparison [32].

SUMMARY

The vitamin B_{12}-factors of twenty-five species of marine algae have been investigated, using *E. coli* and *L. lactis* Dorner as test organisms. The B_{12}-activity of the brown algae (*Phaeophyta*) corresponded to 0.07 μgB_{12} (average value for ten species); of the red algae (*Rhodophyta*, ten species) to 0.27 μgB_{12}; and of the green algae (*Chlorophyta*, four species) to 0.35 μg per gram dry weight. A sample of *Vaucheria dichotoma*, the only alga tested belonging to the division *Chrysophyta*, contained about 2.8 μgB_{12} gram dry weight. The total activity was due to at least four different factors, three of which have been identified as vitamin B_{12} (cyanocobalamin), the main component of pseudovitamin B_{12} and the main component of factor A. The fourth factor showed properties different from those of the hitherto described B_{12}-factors and was therefore given the name vitamin B_{12s}.

Of thirty-four bacteria isolated from seawater and from three different species of marine algae, twenty-four showed a definite ability to produce vitamin B_{12}-factors in concentrations ranging from about 0.005 to 0.14 μgB_{12} per ml fermentation broth. The same type of B_{12}-factors as those present in the algae were produced by these bacteria. Vitamin B_{12s} was found to be produced only during a certain stage of development of the bacterial cultures.

The experimental results do not indicate that seaweeds should be able to synthesize vitamin B_{12}-factors. It seems more likely that the B_{12}-factors are produced by bacteria living epiphytically on the algae and that the factors are subsequently taken up and stored by the plant. No direct evidence has been obtained that the vitamin B_{12}-factors should be needed by the algae, although some data can be interpreted on the basis of a requirement of B_{12}.

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Starch degradation by the α-amylases

III. End products of the salivary amylase action

By Karl Myrbäck and Ebba Willstaedt

With 9 figures in the text

K. H. Meyer *et al.* (1, 2) have stated that all α-amylases have the same action on starch and yield ultimately the same products, *viz.* glucose, maltose and isomaltose. However, there is plenty of evidence to show that the mechanism of action is not the same for all α-amylases (3, 4) and, furthermore, among the end-products of the α-amylase action on starch (or glycogen) appear not only the three sugars mentioned, but also maltotriose (5, 6) and limit dextrins, containing three or more glucose units, linked by 1,6- and 1,4-glucosidic bonds. This was shown especially in the case of salivary amylase. Limit dextrins with 3, 4 or even more glucose units were shown to be stable against salivary amylase action or, at least, to be hydrolyzed at a rate so low that, to all practical purposes, it can be put = 0. These questions have been further investigated and paper chromatography used to characterize the limit dextrins. The results are presented in the following.

Preparation of residual dextrin

850 g potato starch was boiled to a thin paste with about 14 l water. 150 ml filtered saliva and some toluene was added and the mixture was kept at 40° C. pH was adjusted to 6.8 by addition of a few drops of NaOH solution. After six days a small amount of insoluble material was removed in a Sharpless centrifuge. The clear solution contained 57 mg dry substance/ml, corresponding to about 61 mg glucose at complete hydrolysis. The apparent degree of hydrolysis was:

after	6 days	48.2	per cent
»	28 »	51.0	»
»	33 »	51.0	»

Fermentation experiments (7) showed that 10.7 per cent glucose + 71.3 per cent maltose had been formed. The maltose fraction may comprise some maltotriose. 25 ml of the reaction mixture was fermented by baker's yeast, filtered, evaporated to dryness and dissolved in 25 ml water. The reducing power (Bertrand method) corresponded to 33.3 mg glucose, after complete hydrolysis in N sulphuric acid at 100° to 234 mg. The yield of nonfermentable dextrin thus was 15.4 per cent and the average chain length of the dextrins $n = 7$ units.

The whole reaction mixture was reduced to a volume of 10 l and fermented twice with 200 g baker's yeast for 20 hours to remove glucose and at least most of the maltose and maltotriose.

The filtrate was evaporated to 1150 ml, 5 g barium acetate was added and pH brought to 8 by the addition of Ba(OH)$_2$. The precipitate (7 g) was called fraction No. 0. 1000 ml filtrate + 1000 ml alcohol yielded 13 g fraction No. 1; filtrate + 1000 ml alcohol gave 21.1 g fraction No. 2 (all yields are given as dry weight). The filtrate was reduced *in vacuo* to about 300 ml and traces of Ba were removed with sulphuric acid. After filtration and washing 400 ml were obtained. They were precipitated by alcohol:

800 ml alcohol gave	50.9 g	fraction	No. 3
200 » » »	16.4 g	»	No. 4
500 » » »	8.1 g	»	No. 5

The filtrate was reduced to a thin sirup:

200 ml alcohol gave	27.8 g	fraction	No. 6
100 » » »	3.2 g	»	No. 7
300 » » »	1.0 g	»	No. 8

The filtrate was evaporated to a thick sirup:

200 ml alcohol gave	1.6 g	fraction	No. 9
Soluble portion	3.5 g	»	No. 10

Fractions Nos. 0 and 1 contained inorganic material to the larger part and were not further investigated. Fractions Nos. 2–10 contained in total 133 g dextrin or 15.6 per cent of the original amount of starch. This figure agrees well with those reported earlier from this laboratory (4).

The fractions Nos. 2–10 were characterized by the following determinations:

a) Determination of the reducing power, *i.e.* determination of the average chain length;

b) Fermentation experiments with baker's yeast and dried brewer's yeast;

c) Paper chromatography.

a. Average chain length

The reducing power was determined according to the method of Somogyi (8), in most cases also by the iodometrical method of Willstätter and Schudel. The

Table 1.

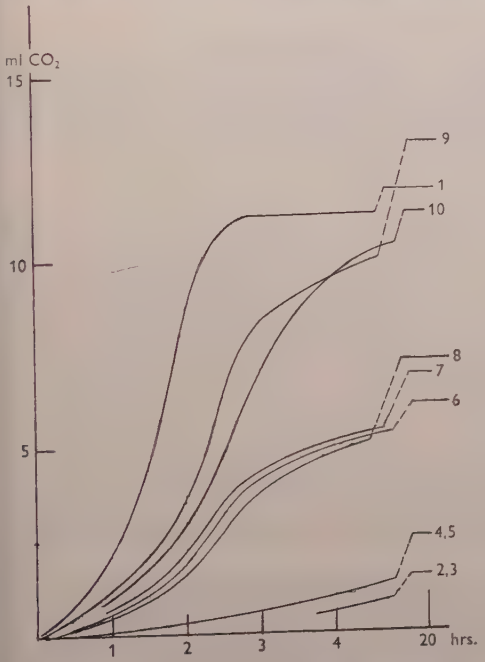
Fraction No.	Yield in percent of starch	Average chain-length n
2	2.48	9
3	5.99	6
4	1.93	5
5	0.85	5
6	3.27	3–4
7	0.38	3
8	0.12	3
9	0.19	3
10	0.41	~2
	15.62	

values agreed well. The values of the average chain length, n, are listed in table 1. In agreement with earlier investigations we find that components with $n = 2$, *i.e.* maltose and isomaltose, can be present only in small amounts. Trisaccharides must be abundant in fractions Nos. 5–9, but the larger portion of the dextrins (fractions Nos. 2–4) has an average degree of polymerisation $= 4$ or more.

b. Fermentability

1) *Baker's yeast.* Fermentation experiments with dextrin fractions Nos. 2–10 are presented in fig. 1. It appears that fractions Nos. 2–3 contain very small amounts of fermentable sugar, 5 per cent at most. More is present in fractions Nos. 4–8, about 10, 10, 27, 32 and 34 per cent, repectively. Fraction No. 9 contains about 60 per cent fermentable substances and fraction No. 10 is fermented almost completely. In all cases glucose is absent. The fermentation is slow but a comparison between the fermentation curves of fractions Nos. 6–10 and that of maltose (fig. 1) shows that some maltose is probably present in these fractions. Part of the carbon dioxide may originate from maltotriose which is fermented slowly by the baker's yeast used in the experiments. This is in agreement with the results of the paper chromatographical investigation of the dextrin fractions (*vide infra*). Isomaltose and higher oligosaccharides of the isomaltose series were not fermented.

2) *Dried brewer's yeast.* This yeast which has been used in a large number of investigations from this laboratory contains practically no living cells (9). The cell



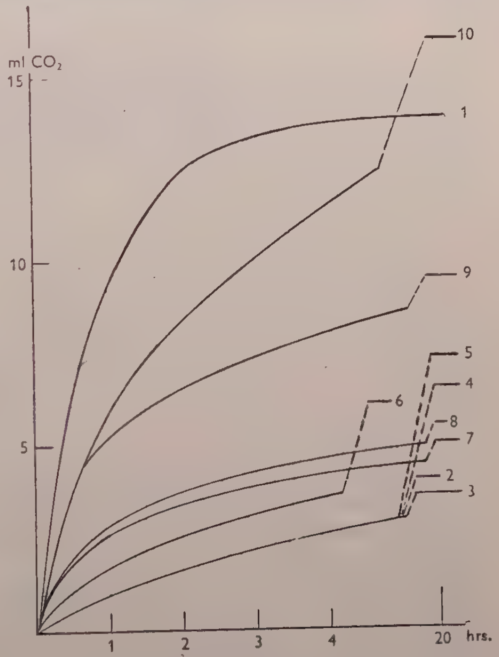


Fig. 1. Curve 1: 50 mg maltose. Curves 2–9: Fractions Nos. 2–9, 90 mg dry weight. Curve 10: Fraction No. 10, 45 mg dry weight.

Fig. 2. Curve 1: 50 mg glucose. Curves 2–9: Fractions Nos. 2–9, 45 mg dry weight. Curve 10: Fraction No. 10, 65 mg dry weight.

wall is completely destroyed; several cell components, *e.g.* cozymase (DPN), are freely soluble, and certain carbohydrates which do not penetrate into the living cell, may be fermented by the dried yeast. It could be shown (10) that a large number of starch dextrins, and even starch itself, are fermented more or less completely. The action of the dried brewer's yeast on our dextrin fractions was determined. A certain amount of each fraction was dissolved in 1.5 ml water, and 0.5 ml 10 per cent phosphate buffer of pH = 6.2 was added. This phosphate concentration and pH have been found to give a maximum rate of glucose fermentation (11). 0.2 g dried yeast was added and the carbon dioxide evolution was measured. A small correction has to be made for the carbon dioxide evolved by the yeast autofermentation. The corrected values are plotted in fig. 2. 50 mg glucose yield 13.8 ml carbon dioxide. Since 65 mg fraction No. 10 have given 16.5 ml CO_2, it follows that practically all saccharides in this fraction are fermented. The rate of fermentation is somewhat lower than that of glucose. Since maltose is fermented by the yeast at practically the same rate as glucose, it follows that at least part of the fermentable saccharides of fraction No. 10 consists of other saccharides than maltose, probably maltotriose and trisaccharides of the isomaltose series. The carbon dioxide evolution from the other dextrin fractions (45 mg) shows that fraction No. 9 is fermented to about 75 per cent, fractions Nos. 4-8 to 40–59 per cent and fractions Nos. 2 and 3 to about 30 per cent. The percentage of substances fermentable by the dried brewer's yeast is considerably higher than that fermentable by the living baker's yeast, *i.e.* the dried yeast ferments at least certain of the saccharides of the isomaltose type.

c. Paper chromatography of the residual dextrins

A few typical experiments are shown in fig. 3. The method used has been described in an earlier paper (12). The horizontal lines in the figure correspond to the R_G-values for the different saccharides, likely to occur in the dextrin fractions. The R_G-values are taken from (12).

It must be remembered that there is no simple relation between the magnitude of a spot and the amount of sugar corresponding to it. The spots have a tendency to grow larger as they move away from the starting line. But even if we disregard the magnitude of the spots and compare *e.g.* the amount of Ag in the spots, we must consider that this amount is not at all proportional to the amount of saccharide in the spot but (more or less) proportional to the reducing power of the saccharide in question. When comparing the spots in our chromatograms, therefore, the upper spots have to be "multiplied" several times to be comparable to the lower, *e.g.* maltose spots.

The following conclusions may be drawn from the chromatograms:

a) All fractions are rather inhomogeneous;

b) glucose is absent from all fractions. This was to be expected since this sugar is fermented rapidly by baker's yeast;

c) fraction No. 2 does not contain any saccharides of the maltose series nor isomaltose nor trisaccharides of the isomaltose series. It contains small amounts of tetrasaccharides of the isomaltose series, somewhat larger amounts of pentasaccharides and relatively large amounts of higher saccharides whose degree of polymerisation cannot be determined from these chromatograms ("spot X"). This is in agreement with the fact that the average DP of fraction No. 2 is as high as about 9;

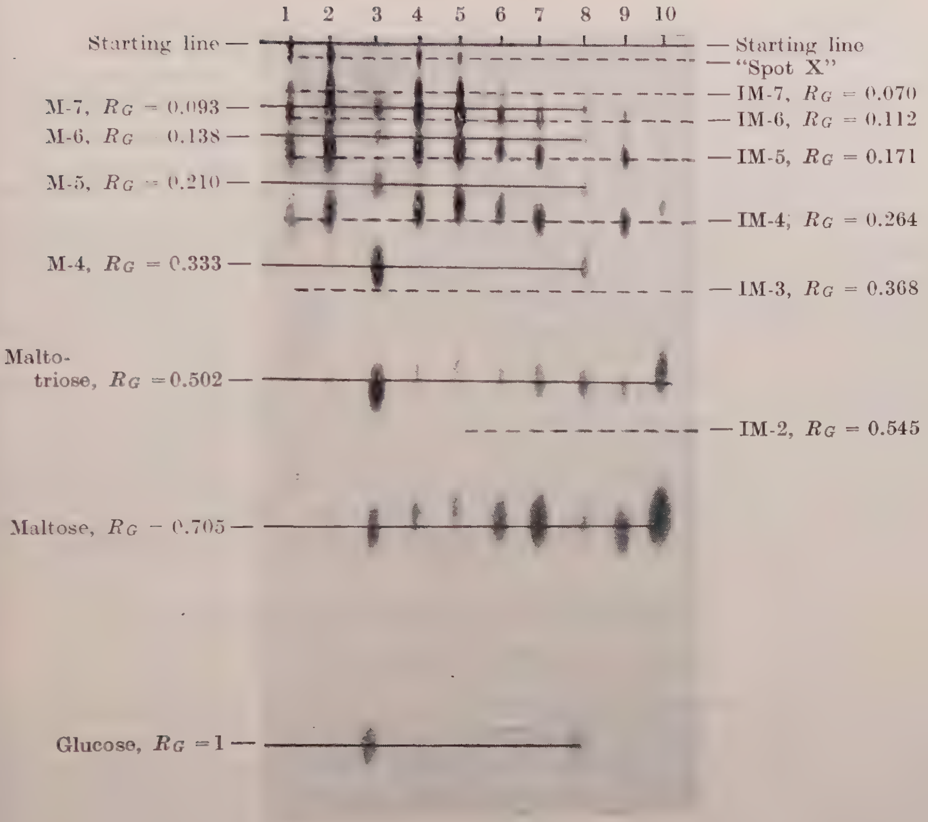

1 2 3 4 5 6 7 8 9 10
Starting line
Starting line
"Spot X"
M-7, RG = 0.093
IM-7, RG = 0.070
M-6, RG = 0.138
IM-6, RG = 0.112
IM-5, RG = 0.171
M-5, RG = 0.210
IM-4, RG = 0.264
M-4, RG = 0.333
IM-3, RG = 0.368
Malto-triose, RG = 0.502
IM-2, RG = 0.545
Maltose, RG = 0.705
Glucose, RG = 1

Fig. 3.

No. 1: Dextrin fraction No. 2.
No. 2: » » No. 3.
No. 3: Sugars of the maltose series
No. 4: Dextrin fraction No. 4.
No. 5: » » No. 5.
No. 6: Dextrin fraction No. 6.
No. 7: » » No. 7.
No. 8: Sugars of the maltose series
No. 9: Dextrin fraction No. 8.
No. 10: » » No. 9.

d) fraction No. 3 contains small amounts of maltose and maltotriose which is understandable since these sugars and especially maltotriose are fermented only slowly by the yeast employed. It contains larger amounts of oligosaccharides of the isomaltose series with 4, 5, 6, and 7 glucose units. "Spot X" is also present;

e) fractions Nos. 4 and 5 appear to have much the same composition as fraction No. 3;

f) in fractions Nos. 6–9 "spot X" is missing, and these fractions do not contain saccharides with more than 7 units;

g) maltose and maltotriose are present in fractions Nos. 6–9;

h) fraction No. 6 contains saccharides of the isomaltose series with 4, 5, 6, and 7 glucose units;

i) fractions Nos. 7 and 8 contain tetra-, penta-, and hexasaccharides of the isomaltose series;

j) fraction No. 9 contains trisaccharides of the isomaltose series, but the nature of the weak spots corresponding to higher saccharides is not clear;

k) in a few other cases weak spots are seen not belonging to the two series of oligosaccharides as defined in (12). It may be that they correspond to isomers of the isomaltose series with different position of the isomaltose linkage.

The result of the chromatographic investigation of the dextrin fractions agrees well with the average DP-values calculated from the reducing power. More than traces of isomaltose have not been detected in any fraction and the amounts of trisaccharides of the isomaltose series are small. These results confirm earlier observations in this laboratory (4) that the limit dextrins of salivary amylase often contain 4 or more glucose units. We have to conclude that these dextrins are stable against the enzyme action or at least that the rate of their hydrolysis is extremely small.

To avoid the complication introduced by the inhibiting action on the enzyme of maltose and glucose we have investigated the action of salivary amylase on isolated dextrin fractions like those mentioned above. Only a very slow action was found and after very long times residual dextrins were still present in large amounts that contained 4 or more glucose units. We have repeated experiments of this kind and used paper chromatography to characterize the products of enzyme action.

Action of α-amylases on fraction No. 3

a. Salivary amylase

0.9 g of dextrin fraction No. 3 was dissolved in 15 ml filtered saliva. pH was adjusted to 6.8 by addition of a trace of sodium hydroxide and the mixture was kept under toluene at 20° C. After suitable times 2 ml samples were evaporated to dryness and dissolved in 1 ml water. 1 ml suspension of baker's yeast was added and the carbon dioxide evolution measured as described before. The fermentation curves are collected in fig. 4. The amounts of glucose and maltose calculated from these curves are found in fig. 5. The maltose values may comprise small amounts of maltotriose, the formation of which, however, does not appear very likely. Obviously maltose is formed from the very beginning of the enzyme action. The amount of maltose rises to a maximum (about 16 per cent) and then decreases, obviously because it is split to glucose. The hydrolysis of maltose by saliva will be analyzed in a forthcoming paper, but we must state already here that at least certain samples of saliva contain small amounts of an enzyme acting on maltose. This contradicts the statement of MEYER (2) that maltase is not present in saliva.

The curves of fig. 5 may be well understood on the assumption that primarily maltose is split off from the substrate and that glucose is formed secondarily from maltose. Let us assume that the substrate is a hexasaccharide that is split by the enzyme in maltose and a tetrasaccharide of the isomaltose series. If these saccharides are designated as c_6, c_4 and c_2, and the rate constant of the hydrolysis is k, then we find the number of molecules of hexa- and tetrasaccharides at the time t from the following formulas (initial concentration of c_6 is put $=1$):

$$c_6 = e^{-kt}; \quad c_4 = 1 - e^{-kt}.$$

Now if the disaccharide (maltose) is split to glucose with the rate constant q, the following equations are valid

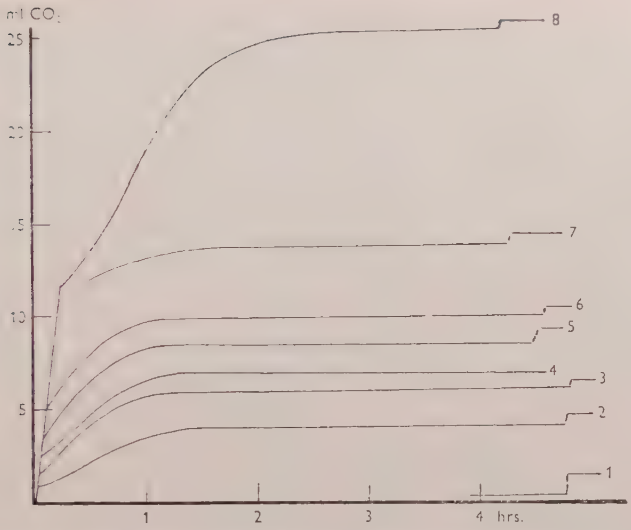


Fig. 4. Fermentations after various reaction times; each sample corresponds to 129 mg theoretical glucose.

Curve 1: Dextrin fraction No. 3.
2: Reaction time 5 days.
3: » » 12 »
4: » » 20 »
5: Reaction time 43 days.
6: » » 100 »
7: » » 200 »
8: 50 mg glucose + 50 mg maltose.

$$\frac{\mathrm{d}\,c_2}{\mathrm{d}\,t} = k\,c_6 - q\,c_2; \quad \frac{\mathrm{d}\,c_1}{\mathrm{d}\,t} = 2\,q\,c_2.$$

Integration gives

$$c_2 = \frac{k}{k-q}\,e^{-qt} - \frac{k}{k-q}\,e^{-kt}$$

and

$$c_1 = \frac{2q}{k-q}\,e^{-kt} - \frac{2k}{k-q}\,e^{-qt} + 2.$$

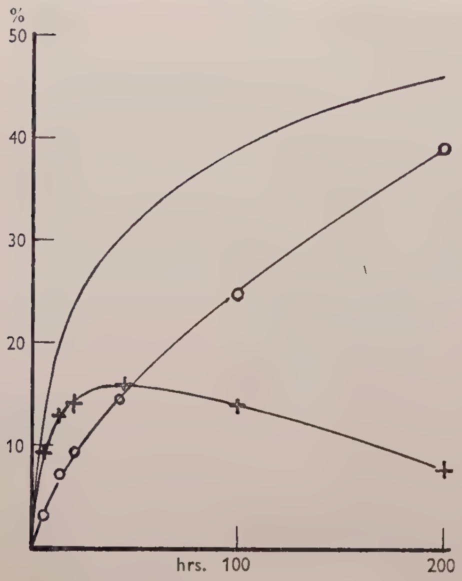
If we put, *e.g.* $q = 0.5\,k$, we get

$$c_2 = 2\,e^{-qt} - 2\,e^{-kt}; \quad c_1 = 2\,e^{-kt} - 4\,e^{-qt} + 2.$$

The yields of maltose and glucose (per cent) calculated from these formulas are represented in fig. 6. The close similarity of figs. 5 and 6 is obvious.

It is emphasized that even if saliva contains maltase and the reaction may proceed as assumed here, it is by no means proven that *all* glucose formed in the experiments originates from maltose.

Fig. 5 shows that the formation of fermentable sugars from dextrin fraction No. 3 has not reached its maximum even after 200 days. On the other hand, it would seem that the final yield of glucose would be something like 50 per cent. Since the starting material (dextrin No. 3) had an average DP of 6 and contained small amounts of maltose and maltotriose, it should be expected that the non-fermentable limit dextrins of the second treatment with saliva would have a DP higher than 3. These limit dextrins, freed from fermentable sugars by baker's yeast, were compared with



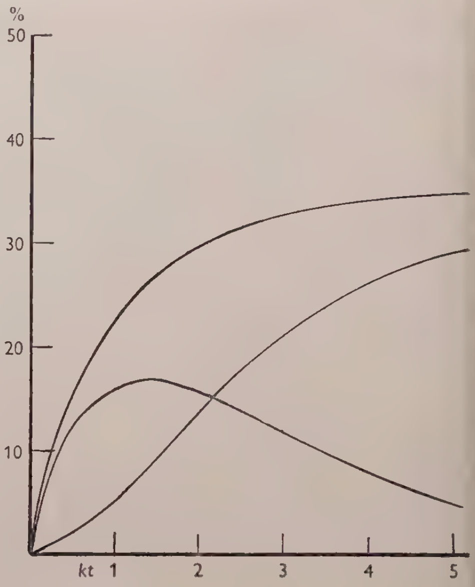


Fig. 5. Yields of maltose (+) and glucose (o) after various reaction times. Upper curve: total yield of fermentable sugar.

Fig. 6. Yields of maltose, glucose and total fermentable sugar, calculated from formulas, p. 449.

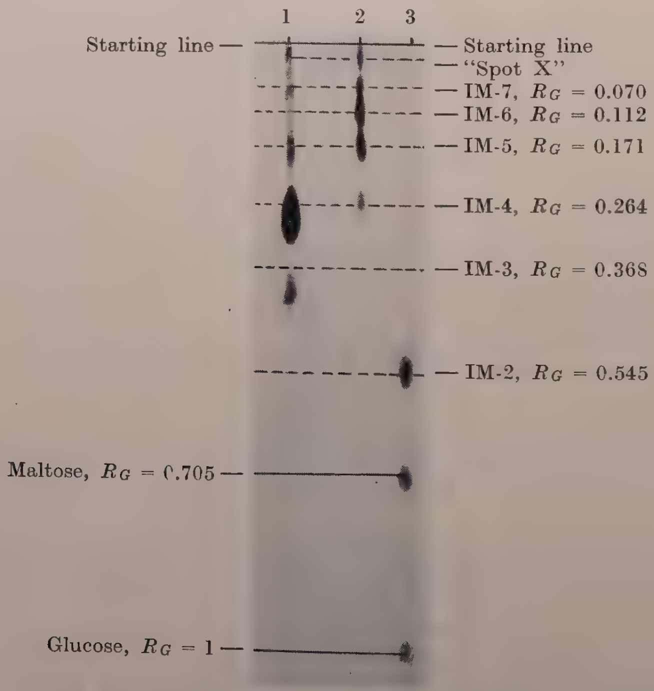

1
2
3
Starting line
Starting line
"Spot X"
IM-7, R_G = 0.070
IM-6, R_G = 0.112
IM-5, R_G = 0.171
IM-4, R_G = 0.264
IM-3, R_G = 0.368
IM-2, R_G = 0.545
Maltose, R_G = 0.705
Glucose, R_G = 1

Fig. 7. No. 1: Dextrin No. 3, after second treatment with saliva. No. 2: Dextrin No. 3, before second treatment with saliva. No. 3: Glucose + maltose + isomaltose.

the original dextrin fraction No. 3 by means of paper chromatography (fig. 7). Whereas the starting material contains small amounts of IM-4 (tretasaccharide of the isomaltose series, cf. (12)), larger amounts of IM-5, IM-6, IM-7 and still higher saccharides, we find that during the second treatment with saliva the fraction IM-4 has increased very considerably whereas the amounts of higher saccharides have decreased strongly. The spot corresponding to IM-6 which, as expected, is the strongest one in the chromatogram of fraction No. 3, has vanished completely, probably by hydrolysis to IM-4 and maltose as assumed above. Thus it would seem that the residual dextrins with a DP higher than 4 are broken down very slowly to fermentable sugars and IM-4. It may be that also this dextrin is not absolutely stable against the enzyme but the rate of hydrolysis must be extremely low. The tetrasaccharide IM-4 can be regarded as a *stable dextrin* of the salivary amylase. It is not at all difficult to understand that a tetrasaccharide of this type is stable against the enzyme since maltotriose which should be a much more "natural" substrate is probably not attacked (5, 6).

The hydrolysis of the higher saccharides (hexasaccharides on the average) to the tetrasaccharide is very slow. The initial rate of this hydrolysis (15 ml saliva, 1 g substrate) is in the neighborhood of 3 per cent in 24 hours, calculated as glucose. If 1 ml saliva is allowed to act on 1 g starch (in 100 ml) this degree of hydrolysis is reached in about one minute, *i.e.* the ratio of the two velocities is certainly smaller than 1 : 72000.

b. Malt α-amylase

1 g fraction No. 3 +15 ml water +0.1 g amylase preparation, partly purified by acetone precipitation. The preparation was free from β-amylase and seemed to have no action on maltose in reasonable times. The mixture was kept under toluene at 20° C. Glucose and maltose were determined by fermentation as above (Table 2).

Maltose was formed comparatively rapidly; the yield reached a maximum of 57 per cent and decreased very slowly. Only small amounts of glucose were formed, and since the yield of maltose decreased after long reaction times we may conclude that at least part of the glucose was formed from maltose by contaminating maltase. If glucose was formed at all from saccharides with 3 or more glucose units, its amount must be quite small. The total amount of fermentable sugar, about 67 per cent of the theoretical, is somewhat higher than in the experiment with salivary amylase. This would intimate that the average DP of the residual dextrins would be lower with the malt enzyme than with saliva. The nature of the degradation products was investigated by means of paper chromatography. A few experiments are shown in fig. 8. The mean R_G-values of the dextrin spots (4 experiments) were 0.510 (weak), 0.378 (strong), 0.255 (strong), 0.169 (weak); 0.116 (very weak); 0.080 (weak). If

Table 2.

Reaction time	Per cent glucose	Per cent maltose
5 days . . .	3	35
12 » . . .	7	55
20 » . . .	8	57
43 » . . .	10.5	54.5
100 » . . .	21.0	46.0

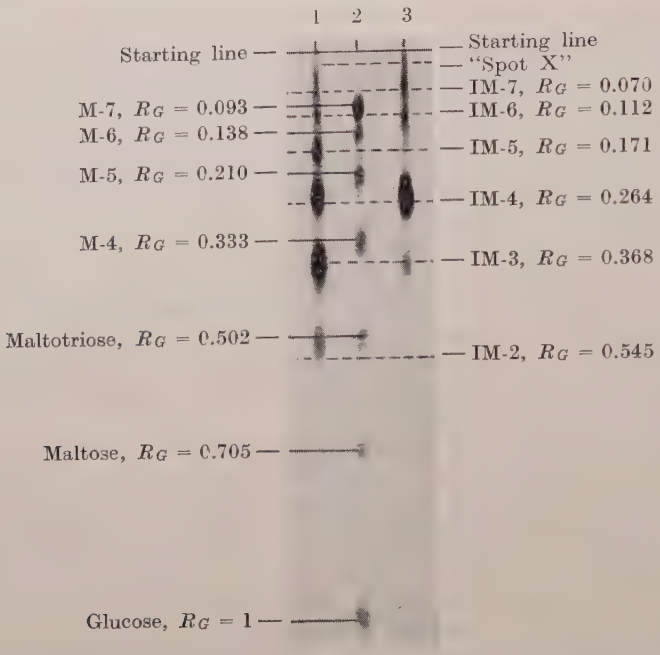

1
2
3
Starting line
M-7, R_G = 0.093
M-6, R_G = 0.138
M-5, R_G = 0.210
M-4, R_G = 0.333
Maltotriose, R_G = 0.502
Maltose, R_G = 0.705
Glucose, R_G = 1
Starting line
"Spot X"
IM-7, R_G = 0.070
IM-6, R_G = 0.112
IM-5, R_G = 0.171
IM-4, R_G = 0.264
IM-3, R_G = 0.368
IM-2, R_G = 0.545

Fig. 8. No. 1: Dextrin No. 3 after treatment with malt α-amylase. No. 2. Oligosaccharides of the maltose series. No. 3: Dextrin No. 6 after second treatment with saliva.

these values are compared to those determined in (12) we may conclude that the main degradation products of the dextrin fraction No. 3 are the tri- and tetrasaccharides of the isomaltose series. Since the yield of fermentable sugar did not increase markedly from 12 to 100 days reaction time, it seems permissible to designate the two saccharides as *stable dextrins*. Only small amounts of isomaltose ($R_G = 0.545$) were formed. No or practically no liberation of glucose from tri- and tetrasaccharides could be observed, showing again that the assumption of Meyer *et al.* (1, 2) concerning the last stages of α-amylase action are not correct, not even in the case of malt α-amylase.

Action of amylases on fraction No. 6

a. Salivary amylase

The experimental conditions were the same as in the experiment with fraction No. 3. Glucose and maltose were determined by fermentation with baker's yeast as above (Table 3).

The amount of maltose increases a little but decreases again as in the experiment with fraction No. 3, obviously because maltase is present in small quantities. The yield of glucose increases steadily, at least partly because of the maltase action. However, it may well be that glucose is also split off from higher saccharides. The low maximum yield of maltose is understandable since the average DP of fraction No. 6 is as low as 3.

The paper chromatogram (No. 6 in fig. 3 and No. 3 in fig. 9) shows the presence of the following non-fermentable saccharides: IM-4, IM-5, IM-6 and IM-7. New

Table 3.

Reaction time	Salivary amylase		Malt α-amylase	
	Per cent glucose	Per cent maltose	Per cent glucose	Per cent maltose
5 days	3.5	24.0	3.4	47.0
12 »	7.1	24.0	6.8	51.0
20 »	11.5	24.0	—	—
43 »	20.5	21.2	10.0	52.0
100 »	44.3	17.7	65	

chromatograms were made after the second treatment of dextrin No. 6 with saliva and fermentation (No. 3 in fig. 8). Glucose, maltose and maltotriose are absent, obviously because they have been fermented away. Among the saccharides of the isomaltose series we find small amounts of IM-3, IM-6 and IM-7; the pentasaccharide IM-5 is absent but the amount of IM-4 has increased very considerably. In agreement with earlier experiments we thus find that the tetrasaccharide of the isomaltose series is a very if not completely stable dextrin ("limit dextrin"). It should be noted that practically no isomaltose, IM-2, has been formed. The R_G-value of the tetrasaccharide is 0.259 (mean value of 7 experiments) which agrees very well with that (0.264) found in (12).

b. Malt α-amylase

The experiment was carried out as the corresponding one with dextrin fraction No. 3. Fermentable sugars in table 3. Chromatograms were prepared after fermenta-

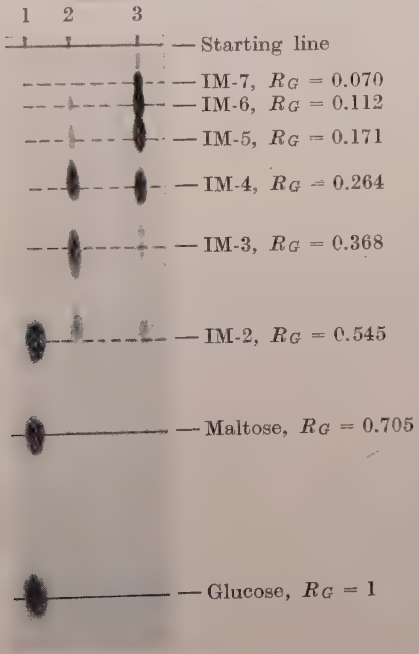


Fig. 9. No. 1: Glucose + maltose + isomaltose. No. 2: Dextrin No. 6 after treatment with malt α-amylase. No. 3: Dextrin No. 6 after fermentation with baker's yeast.

tion (No. 2 in fig. 9). It appears that the dextrin IM-5 and higher members of the isomaltose series have practically vanished. Large amounts of IM-4 and IM-3 are present together with smaller amounts of isomaltose, IM-2. The high stability of IM-4 and IM-3 is evident also in this experiment. If isomaltose is formed by the amylase itself, which cannot be stated with certainty on the basis of these experiments, it is quite clear that the rate must be extraordinarily low.

SUMMARY

The non-fermentable dextrins produced by intensive treatment of starch paste with saliva have been isolated and roughly separated into ten fractions by ethanol precipitation. These fractions have been characterized by determination of the average degree of polymerization, determination of fermentability and by paper chromatography.

Isomaltose was not found in any of the fractions and only small amounts of trisaccharides of the isomaltose series.

Maltotriose (which is fermented slowly by the yeast used for removal of fermentable sugars) was present in some fractions.

Oligosaccharides of the isomaltose series, containing 4, 5, 6, 7, and in some cases even more glucose units were present as chief components.

Treatment of the higher saccharides (five and more glucose units) with large amounts of saliva for long periods of time resulted in an extremely slow hydrolysis yielding fermentable sugars and a tetrasaccharide of the isomaltose series. This saccharide can probably be regarded as a true stable dextrin of salivary amylase.

Treatment of the same dextrin fraction with malt α-amylase yielded the same products, but a trisaccharide of the isomaltose series was also formed, possibly also some isomaltose. The hydrolysis rate of the tetra- and trisaccharides must be extraordinarily low compared to that of starch.

Saliva, or at least certain samples of saliva, contain traces of maltase.

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Enzyme in neoplastischen Geweben

Von Hans von Euler

Mit 3 Figuren im Text

Die Fortschritte der Enzymologie im letzten Dezennium beruhen nicht auf einem das ganze Gebiet befruchtenden experimentellen Ergebnis oder einer umfassenden Theorie; sie bestehen vielmehr in der starken Erweiterung des älteren Tatsachenmateriales durch den Nachweis der enzymatischen Natur mancher biochemischen Reaktionen, in der Isolierung der betr. Enzyme und in der Ermittlung der Struktur ihrer Wirkungsgruppen (Co-Enzyme). Dadurch kann nunmehr auch eine grössere Anzahl der in Tumoren und in cancerösen Organismen verlaufenden Vorgänge bestimmten Enzymen oder Enzymkomplexen zugeordnet und in biochemischer Hinsicht aufgeklärt werden.[1]

Die aktuellen Probleme und Aufgaben der modernen Enzymologie sind auch für die Tumorforschung von wesentlicher Bedeutung. Diese Probleme betreffen besonders die Enzyme des Aufbaues (und stehen deswegen mit dem energietransportierenden ATP und den energiereichen Phosphaten, Phospho-enol-brenztraubensäure u. a. in nächster Beziehung) und betreffen die Rolle, welche Enzymkomplexe (Enzymoide) beim Wachstum normaler und neoplastischer Gewebe spielen.

Enzymsysteme des oxydativen Stoffwechsels

Die Enzyme des Glycogen-Abbaues und ihre Funktionen

Seit etwa 1936 weiss man[2], dass der Abbau des Glycogens überwiegend nicht mit dessen Hydrolyse, also nicht durch eine Glycogenase einsetzt, sondern mit einer Phosphorylierung durch eine Phosphorylase[3]. Dieselbe enthält, wie sich später zeigte, Adenylsäure als prosthetische Gruppe und wird durch Adenosintriphosphat (ATP) aktiviert. Die Phosphorylase kann aus frischem Muskelextrakt in sehr hoher Aktivität gewonnen werden. Beim Abbau des Glycogens entstehen zunächst

Glucopyranose-1-phosphat, Cori-Ester, sowie
Glucopyranose-6-phosphat, Robison-Ester und schliesslich
Fructofuranose-1,6-diphosphat, Harden-Young-Ester.

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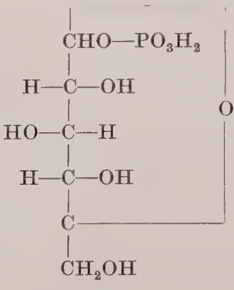
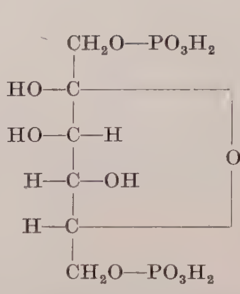
[1] Vgl. Fr. Pentimalli, Problemi del Servicio Sociale, ed. G. Petrilli, p. 201, Roma 1953.

[2] J. K. Parnas u. T. Baranowski, C. r. Soc. Biol. 120, 307; 1935.

[3] Mit „Phosphorylase" haben wir in Übereinstimmung mit Quastel unsere frühere Bezeichnung Heterophosphatase (Z. physiol. Chem. 235, 128; 242, 24; 1936) ersetzt, die wir ursprünglich für das Enzym der Umesterung von ATP mit Hexose angewandt hatten. Da vermutlich verschiedenen Umesterungsreaktionen durch verschiedene Phosphorylasen katalysiert werden, soll der Name des Phosphatakzeptors vor dem Namen Phosphorylase gesetzt werden. z. B. Hexose-Phosphorylase für die Umesterung ATP-Hexose.



CORI-Ester



HARDEN-YOUNG-Ester

Man verdankt CARL F. CORI und GERTY T. CORI[1] grundlegende Arbeiten über den normalen enzymatischen Abbau des Glycogens. Sie stellen diesen Abbau folgendermaassen dar[1]:

$$\begin{tikzcd}
 & \text{Muskel-Glycogen} + H_3PO_4 \arrow[d, shift right] & \\
 & \text{Glycose-1-phosphat} \arrow[u, shift right, "\text{Phosphorylase}"'] \arrow[dl, "\text{Phosphatase}"'] \arrow[dd, shift right] & \\
\text{Glucose} + H_3PO_4 & & \\
 & \text{Glycose-6-phosphat} \arrow[uu, shift right, "\text{Phosphoglucomutase}"'] \arrow[ul] \arrow[r] & \text{Milchsäure}
\end{tikzcd}$$

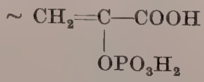
Muskel-Phosphorylase wurde von G. T. CORI und GREEN[1] in krystallisierter Form erhalten.

Phospho-gluco-mutase, isolierbar aus Muskeln, vermittelt die gegenseitige Verwandlung der Glucose-1-phosphorsäure und der Glucose-6-phosphorsäure unter Mitwirkung der 2-wertigen Ionen Mg, Mn oder Co; sie wird dementsprechend durch Fluorid gehemmt. Bei dieser Umwandlung fungiert Glucose-1,6-diphosphat als Co-Enzym.

Durch eine *Isomerase* wird Glucose-6-phosphorsäure nach EMBDEN und NEUBERG in Fructose-6-phosphorsäure verwandelt, was übrigens (unvollständig) auch ohne Enzym (entspr. der LOBRY DE BRUYN-Reaktion) unter intermediärer Endolisierung gelingt.

Das für die Phosphorylierung und viele damit verknüpften Reaktionen so wichtige, als Energie-Überträger fungierende energiereiche *Adenosintriphosphat*, ATP, wird durch die Adenosintriphosphatase (ATP-ase) in Phosphat und ADP (Adenosindiphosphat) gespalten: ATP → ADP + H_3PO_4.

Durch Vermittlung von ATP entstehen Energie-reiche P-Verbindungen (mit ~ bezeichnet) u. a. Phospho-enol-brenztraubensäure



$$\sim CH_2{=}\underset{\displaystyle OPO_3H_2}{\underset{|}{C}}{-}COOH$$

Auch der enzymatische Abbau der freien Glucose im Tierkörper beginnt mit der Phosphorylierung, und zwar mit der Überführung des Phosphatrestes von Adenosintriphosphat auf die Glucose durch *Hexokinase*.

Die Hexokinase wird mit anderen Enzymen der Phosphatübertragung in Zusammenhang mit dem Abbau der freien Glucose behandelt. Die übrigen Enzyme des Zuckerabbaues, Phosphohexose-isomerase, Aldolase, Enolase, Phospho-glyceromutase sollen hier nur insoweit besprochen werden, als sie an charakteristischen Unterschieden zwischen normalen und cancerösen Organen und Geweben beteiligt sind.

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[1] C. F. CORI u. G. T. CORI, Proc. Soc. exp. Biol. a. Med. 34, 702; 1936 und G. T. CORI u. A. A. GREEN, J. biol. Chem. 151, 36, 119; 1937.

Mit dem phosphorylierenden Angriff des Glycogens und seiner Spaltprodukte ist der oxydo-reduktive Kohlenhydratabbau stöchiometrisch verknüpft. Eine solche Kuppelung des Zuckerabbaues mit der Bildung des Hexosephosphates liess sich für Hefe der schon 1906 von Harden aufgestellten Gärungsgleichung entnehmen.

Um die Übersicht über dieses ausserordentlich grosse Gebiet enzymatischer Vorgänge zu erleichtern wollen wir uns im Folgenden möglichst streng auf unser Ziel konzentrieren, nämlich auf den Vergleich des Kohlenhydratabbaues im normalen und im cancerösen Tierkörper.

Dass *Sarkom-Extrakte* instande sind, zugesetztes Glycogen zu phosphorylieren konnten Euler und Schlenk[1] sowie Euler und Bauer[2] aus ihren 1937 ausgeführten Versuchen schliessen.

Mit der Methylenblau-Methodik fanden Euler, E. Bauer und Forsman[3] in Jensen-Sarkomen eine stark wirksame Robisonester-dehydrase. Dabei konnte Glycogen zum Unterschied von Glucose als Wasserstoffdonator verwendet werden. Da nach dieser Untersuchung Robisonester durch den Jensensarkom-Extrakt energisch dehydriert wurde, so lag der Schluss nahe, dass auch in diesem Falle Glycogen über ein Glucosephosphat abgebaut wird.

Auch weiterhin ist wiederholt untersucht worden, in welchem Grad in Tumoren der phosphorylierende Glycogenabbau vorherrscht.[4] Nach Kobayasi[5] können Tumorauszüge Hexosediphosphorsäure in Phosphoglycerinsäure umwandeln, wenn man dem System Adenosintriphosphorsäure zufügt. Durch diese Auszüge wurde auch Glycogen in Hexosediphosphat umgesetzt, wie durch das Vorkommen der Hexosekinase im Tumorgewebe verständlich wird.

Besonders wertvolle Aufschlüsse über Enzymwirkungen beim Glycogenabbau in Sarkomgeweben hat Otto Meyerhof in den letzten Jahren vor seinem Tod geliefert. Um eine schnelle und konstante Glykolyse freier Zucker in Abwesenheit von Phosphatdonatoren zu erreichen muss die Aktivität der Adenosintriphosphatase (ATP-ase) im Verhältnis zum Phosphatumsatz das doppelte der Hexokinase-Aktivität betragen. Nun fand Meyerhof in Homogenaten und Extrakten aus Rattensarkomgewebe einen sehr grossen Überschuss an ATP-ase gegenüber Hexokinase. Im Gegensatz zu Hirngewebe scheint darin die ATP-ase zum grossen Teil nicht an Zellen gebunden zu sein und konnte deswegen nicht abzentrifugiert werden. Dadurch wurde ATP schneller abgebaut als regeneriert (Arch. Biochem. 21, 1).

Ausserdem wurden Hexosekinase und ATP-ase untersucht. Bemerkenswert ist die Hemmung der ATP-ase von Tumorgewebe durch Toluol, Octylalkohol, Decylalkohol und Laurylalkohol. Entsprechende Hemmungen konnten an ATP-ase anderer Gewebe nicht beobachtet werden. Andere Enzyme der Glycolyse waren nicht beeinflusst, so dass in Homogenaten und Extrakten aus Tumorgeweben eine deutliche Glykolyse freier Zucker einsetzte. Durch Na-azid wurde ein ähnlicher

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[1] Euler u. Fr. Schlenk, Arkiv f. Kemi etc. 12 B No. 49; 1938.

[2] Euler, Erwin Bauer, u. K. Lundberg, Z. physiol. Chem. 255, 89; 1938. Naturwiss. 26, 235; 1938. — Euler, Arkiv f. Kemi etc. 13 B No. 11; 1939.

[3] Euler, Fr. Schlenk u. N. Forsman, Naturwiss. 26, 11; 1938.

[4] Holmes, Biochem. J. 34, 926; 1940. — A. B. Novikoff, Van R. Potter u. G. A. Le Page, Cancer Res. 8, 203; 1948.

[5] T. Kobayasi, J. Biochem. Chemistry (Japan) 32, 289; 1940.

Effekt erhalten. Mit Glucose wurden so Q_{la}-Werte (durch Milchsäure ausgetriebene mm³ CO_2/Std/1 mg trockenes Tumorgewebe) von 40–60 gewonnen, die den Werten in lebenden Tumorgewebe ähnlich waren.

In Fortsetzung dieser Untersuchung[1] wurden die Tumorhomogenate von Ratten (transplantierte Sarkome) mit denen von Mäusen (Carcinome und Sarkome) verglichen: Die Messung der Hexokinase-Aktivität ergab in Mäusecarcinom im Gegensatz zu Rattensarkom bei hoher Zuckerkonzentration gleiche Phosphatübertragungen bei Glucose und Fructose. Bei niedriger Zuckerkonzentration (unter 0,05 %) war in Rattensarkom die Phosphatübertragung bei Fructose sehr gering, während sie in Mäusecarcinom noch 50 % derjenigen bei Glucose betrug. Je stärker die Phosphatübertragungen waren, um so stärker wurde die Abspaltung von anorganischen Phosphat gehemmt. Nimmt man an, dess *zwei* Hexokinasen existieren, nämlich Glucokinase und Fructokinase, so würde das Carcinom relativ mehr Fructokinase enthalten als das Sarkom.

Bei Untersuchung der Aldolase-Aktivität wurde Q_p für Tumorhomogenate zwischen 110 und 180 gefunden, während am Muskel Q_p etwa 1600 betrug.

Dehydrasen

Die Dehydrasen oder Dehydrogenase, deren Funktion darin besteht, dass sie Wasserstoff von einem Substrat (Donator) auf eine Akzeptor-Substanz übertragen, zeigen, wie schon T. THUNBERG betont hat, einen sehr hohen Grad von Substrat-Spezifität, und man hat demgemäss eine sehr grosse Anzahl von Dehydrasen zu unterscheiden. Die Mehrzahl der „Holo-Dehydrasen" lässt sich relativ leicht in ein als „Apo-enzym" fungierendes Protein und eine Wirkungsgruppe (katheptische Gruppe) Co-Dehydrase spalten. Man kennt zwei verschiedene Co-Dehydrasen: Co-Dehydrase I, meist Cozymase genannt, wurde von EULER und SCHLENK als Diphospho-pyridinnukleotid erkannt; Co-Dehydrase II von O. WARBURG als Triphospho-pyridinnukleotid.

Die Spezifität der Dehydrasen und die Natur der ihnen zugehörenden Co-Dehydrase wird aus folgenden Auszug aus einer Tabelle des Verfs. ersichtlich:

Dehydrase d. Reaktion	Herkunft d. Enzyms	Co-Dehydrase
Alkohol-Acetaldehyd	Leber, Hefe	I (Cozymase)
Milchsäure-Brenztraubens.	Herzmuskel	I »
Äpfelsäure-Oxalessigsäure	Herzmuskel	I »
α-Glycerinald.-phosphors.-Phosphoglycerins.	Hefe	I »
Glycerinphosphors.	Muskel	I »
Glucose. Glucons.		I oder II
Glutamins. Iminoglutars.	Tierische Organe	I

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[1] O. MEYERHOF u. J. R. WILSON, Arch. of Biochem. 21, 22; 1949. Siehe auch die früheren Arbeiten von O. MEYERHOF und KIESSLING (Biochem. Z. 276, 239; 1934) über Phosphoglycerinsäuren sowie von K. LOHMANN u. MEYERHOF (Biochem. Z. 273, 60; 1934) über die Umwandlung Phosphoglycerinsäure—Brenztraubensäure.

[2] EULER, Ergebn. d. Vitamin- und Hormonforschung 1, 159, 1938.

[3] EULER, E. ADLER, T. STEENHOFF-ERIKSEN, Z. physiol. Chem. 248, 227; 1937. — F. SCHLENK, Naturwiss. 25, 668; 1937. — R. VESTIN, Naturwiss. 25, 667; 1937. — EULER, u. E. BAUER, Chem. Bot. 71, 411; 1938.

Wie Versuche des Stockholmer Institutes gezeigt haben, können die beiden Co-Dehydrasen enzymatisch in einander übergeführt werden.

Der energieliefernde oxydative Kohlehydratabbau[1] wird eingeleitet durch die *Triosephosphorsäure*, die unter Mitwirkung von Co-Dehydrase I (Cozymase) und der Diaphorase dehydriert wird.

Den Nachweis der Triosephosphorsäure als Zwischenprodukt der alkoholischen Gärung erbrachte Ragnar Nilsson[2], dem es gelang, die optisch aktive linksdrehende Form der *Glycerinsäure-Phosphorsäure* aus den Gärungs-system zu isolieren. Einige Jahre später konnten Embden, Deutike und Kraft[3] sowie Meyerhof und Kiessling[4] die gleiche Säure als Stoffwechselprodukt des Muskels nachweisen.

Die Tatsache dass die Wasserstoffübertragung die wesentliche Funktion der Cozymase ist und der Mechanismus der dabei in Betracht kommenden Reaktionen der Atmung und Gärung konnten von Euler, Adler und Hellström vollständig klargestellt werden. Es konnte nämlich gezeigt werden:

1) dass die Cozymase Co selbst durch Wasserstoffaufnahme in Dihydro-cozymase, CoH_2, übergeht[5], welche als Baryumsalz isoliert wurde,

2) dass die Dihydro-cozymase durch Vermittlung eines Enzyms, der Diaphorase, zurückoxydiert wird[6].

Im Verlauf unsere Untersuchungen über Dehydrasen und über den Mechanismus der enzymatischen Dehydrierung hat es sich zweckmässig gezeigt, zwischen Akzeptorsystem und Donatorsystem zu unterscheiden. Als Beispiel führen wir zwei besonders wichtige Oxydoreduktionssysteme an, nämlich das System der Milchsäurehydrase (Lacticodehydrase D^L) und der Äpfelsäurehydrase (Malicodehydrase D^M).

$$\text{Brenztraubensäure} + D^L - \text{CoH}_2 \rightarrow \text{Milchsäure} + D^L - \text{Co}.$$
$$\text{Oxalessigsäure} + D^M - \text{CoH}_2 \rightarrow \text{Äpfelsäure} + D^M - \text{Co}.$$

Die Wirksamkeit der beiden Dehydrasesysteme ist von Bedeutung für die Steuerung des Substrat-Wasserstoffs in der Zelle, soweit der Wasserstoff den beiden Systemen gemeinsamen Überträgersubstanz, der Cozymase — zugeführt worden ist. In Hinsicht auf Tumoren haben wir nun in Erwägung gezogen, dass die Apodehydrase-Moleküle im Tumor gegenüber dem normalen Gewebe irgendwie verändert sind, was eine veränderte Kinetik der Enzymreaktion veranlassen könnte. Es wurde deshalb untersucht, ob im Tumor eine Störung in den genannten Dehydrasesystemen vorliegt im Sinne eines Übergewichtes der Lacticodehydrase gegenüber der Malicodehydrase. Dies trifft für diese Fälle nicht zu.

Das System der Lacticodehydrase des Jensensarcoms unterscheidet sich bezüglich des Gleichgewichtes nicht von den System der entsprechenden Apodehydrase aus Muskel, und das Gleiche gilt von der Malicodehydrase.

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[1] Was die energieliefernde Reaktion des Tumorwachstums betrifft, so hat schon 1933 P. Rondoni und dann eingehender H. Druckrey (Klin. Wochenschr. 1936) auf die Unzulänglichkeit der reinen Glycolyse-Theorie der Tumorenentstehung hingewiesen, und in ähnlicher Weise hat sich u. a. auch R. Deotto (Tumori, 22, 77, 1936) ausgesprochen, der seine Versuche während der krebserregenden Behandlung (Teerpinselung) angestellt hat. Seine Versuche ergaben keinerlei Anhaltspunkte für eine Anaerobiose.

[2] R. Nilsson, Sv. Kem. Tidskr. 41, 169; 1929. — Arkiv f. Kemi etc. 10 A, 7; 1930. Euler u. R. Nilsson, Skand. Arch. Physiol. 59, 201 (1930).

[3] G. Embden, H. J. Deutike u. G. Kraft, Klin. Woch. 12, 213; 1933.

[4] O. Meyerhof u. W. Kiessling, Biochem. Z. 264, 40; 1933.

[5] Euler, E. Adler u. H. Hellström, Sv. kem. Tidskr. 47, 290; 1935.

[6] E. Adler, Euler u. H. Hellström, Arkiv f. Kemi etc. 12 B No. 38; 1937.

Auch durch Zusatz von Hemmungsstoffen konnten Unterschiede zwischen den Dehydrasesystemen des Muskels und des Jensensarkoms nicht hervorgerufen werden.

Die Besonderheiten des Stoffwechsels im Tumor sind noch nicht vollständig aufgeklärt und demgemäss sollen die Wirkungen der enzymatischen Phosphopherasen hier nicht näher besprochen werden. Indessen sei hier schon im Anschluss an die vom Verfasser früher entwickelten Vorstellungen über *Enzymoide*[1] auf den Mechanismus *zusammenwirkender* Enzymsysteme bzw. auf den Reaktionsverlauf in Enzymkomplexen, z.B. Cyclophorasen, hingewiesen.

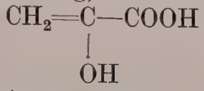
Die Verbindung eines Substrates mit den Holodehydrasen kommt durch die Apodehydrasen zustande, Proteine die bis zu einem gewissen Grad gereinigt werden konnten. Man hat bisher in der Enzymforschung Organextrakte soweit zu fraktionieren versucht, als sie eine einzige Reaktion katalysieren, bzw. auf ein einziges Substrat ansprechen. Der Umstand, dass man noch keine genau definierten und reproduzierbaren Enzymkomplexe kennt, in welchen zwei oder mehrere Wirkungen in konstantem Verhältnis stehen, beruht wohl darauf, dass man solche Komplexe nie aufgesucht sondern im Gegenteil vermieden hat.[2]

Wesentlich für den Ablauf der Oxydationsprozesse ist der Abstand, in welchem die Teilenzyme an der Unterlage haften, somit die Reihenfolge, in welcher die einzelnen Enzyme im Enzymkomplex so angeordnet sind, dass ein Enzym sein Reaktionsprodukt dem benachbarten Enzym als Substrat überliefert.

Beim phosphorylierenden Abbau des Glycogens über Hexose-phosphorsäure, Phosphoglycerinsäure und Phosphobrenztraubensäure entsteht als zentrales Stoffwechselprodukt die *Brenztraubensäure*. Ihre wichtigsten Umsatzreaktionen sind die folgenden:

a. Reversible Hydrierung zu Milchsäure durch Lactico-dehydrase,

b. Decarboxylierung durch die Carboxylase deren Wirkungsgruppe die von AUHAGEN[3] entdeckte Co-Carboxylase als Aneurin-pyrophosphat erkannt ist,

c. Enolisierung, durch welche die in der Regel überwiegende Ketoform in die Enolform  $CH_2{=}C(OH){-}COOH$ übergeht,

d. Aminierung, beginnend mit Bildung der Imino-propionsäure, Ausgangsstoff für zahlreiche Umaminierungen[4],

e. Einstellung des Gleichgewichtes bei der oxydativen Decarboxylierung mit *Co-Enzym A* (CoA) unter Bildung der „aktivierten Essigsäure“[5], welche mit Oxalessigsäure in Citronensäure übergeht; LIPMANN. Die dazu erforderliche Energie wird durch die dehydrierende Decarboxylierung der Brenztraubensäure oder durch die Vermittlung der Adenosintriphosphorsäure gewonnen. An diesem interessanten, enzymchemisch von OCHOA aufgeklärten Vorgang, sind zwei Enzyme beteiligt, ein aktivierendes und ein kondensierendes.

f. Aufnahme von CO_2 nach WOOD u. WERKMAN[6] sowie KREBS und EGGLETON[7].

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[1] EULER, 2 Congrès international de Path. comp.

[2] EULER, Erg. d. Vit.- und Hormon-Forsch. 1, 159; 1937.

[3] E. AUHAGEN, Z. physiol. Chem. 204, 149; 1932 u. 209, 20; 1932. — Biochem. Z. 258, 330; 1933.

[4] A. E. BRAUNSTEIN u. KRITZMANN, Nature 140, 503 (1937). — EULER, ADLER, GÜNTHER und N. B. DAS, Z. physiol. Chem. 254, 61; 1938.

[5] F. LIPMANN, J. Biol. Chem. 187, 757; 1951, 191, 365; 1951.

[6] H. G. WOOD, C. H. WERKMAN, Biochem. J. 32, 1262; 1938. — Advances in Enzymologi, 2, 135; 1942.

[7] H. A. KREBS u. L. V. EGGLETON, Biochem. J. 37 u. 38; 1943/44.

Wir sind nun an der letzten Stufe des Oxydativen Kohlehydratabbaues angelangt, am *Zitronensäure-cyklus*, der mit der Oxalessigsäure zu Zitronensäure und über Ketoglutarsäure, Aconitsäure (Martius) zur *Bernsteinsäure* führt. Diese wird durch die *Succino-dehydrase* zu Fumarsäure dehydriert, die ihrerseits an das Cytochromsystem angeschlossen ist.

Reduktion von Methylenblau durch Lebergewebe normaler und Jensensarkome tragender Ratten und durch Hepatome

A. Lebergewebe von normalen und Jensensarkomen tragenden Ratten

(Inga Pettersson, K. Lundberg und I. Gernow)

Zur Charakterisierung des physiologischen Zustandes der Leber von Ratten, die während des Wachstums implantierter Jensensarkome gewisse Zusätze zur normalen Diät erhalten haben, wurden in mehreren Versuchsreihen in den Jahren 1937–1941 Dehydrasen-Systeme der Leber untersucht.

Die mit I. Pettersson, K. Lundberg, I. Gernow und L. H. P. Pulkki durchgeführten Versuchsreihen bezogen sich (ausser auf Katalase) hauptsächlich auf Citroco-Dehydrase, Lactico-Dehydrase und Succino-Dehydrase.

Was die Versuchsmethodik betrifft, die sich im Wesentlichen der von T. Thunberg[1] und von G. Ahlgren[2] ausgearbeiteten anschliesst so kann auf die etwa gleichzeitigen in diesem Arbeitskreis ausgeführten Untersuchungen von Adler, Günther und B. Andersson[3] verwiesen werden. Das frische Leber-Material wurde — auf 0° bis 5° abgekühlt — mit Sand fein verrieben, durch Collodium-Filter gegen

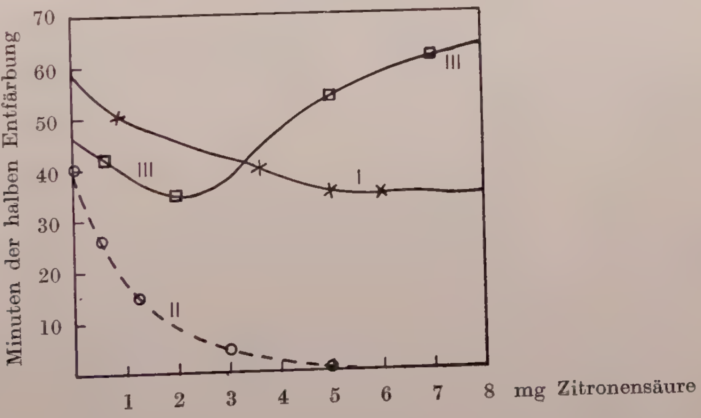

Fig. 1. K. Lundberg.

I. Normales, frisches Lebergewebe.
II. Lebergewebe einer Sarkom-tragenden Ratte 3 Wochen nach Transplantation.
III. Dasselbe Lebergewebe, Zusatz von 5 γ Chinonimid.

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[1] T. Thunberg, Skand. Arch. f. Physiol. 40, 1; 1920.
[2] G. Ahlgren, Akad. Abh., Lund, 1925.
[3] B. Andersson, Gradual-Abh., Stockholm 1938.

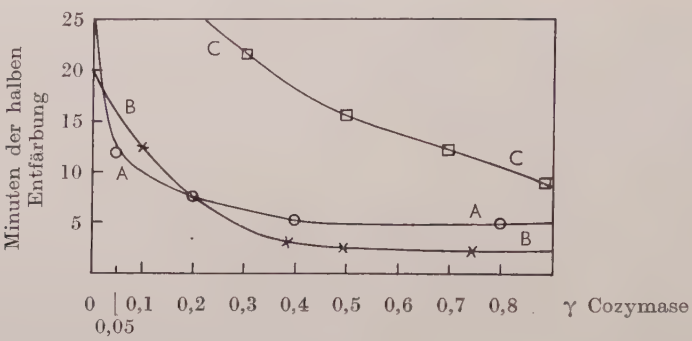

Fig. 2. I. Gernow.

A. } Normales Lebergewebe { 1,2 mg Lactat.
B. } { 2,6 mg Zitrat.
C. Lebergewebe einer sarkomtrag. Ratte, 18 Tage nach Transpl. 1,2 mg Lactat.

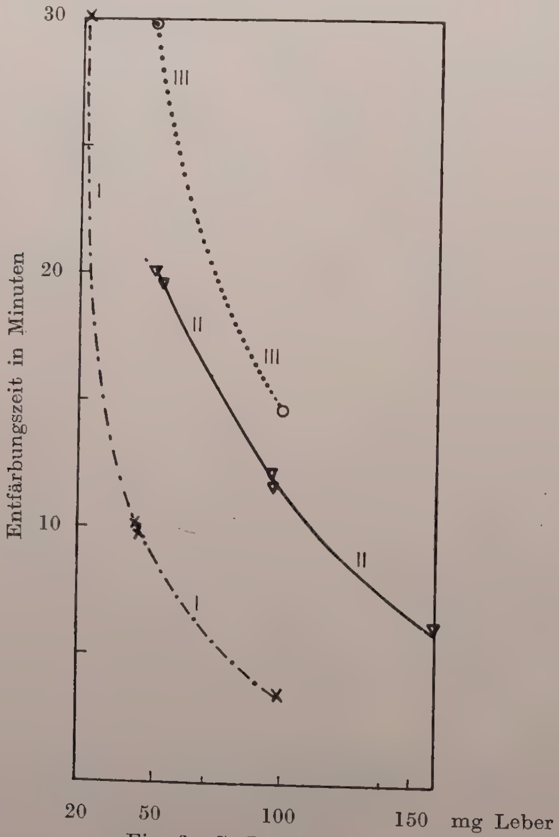

Fig. 3. G. Hanshoff.

BD I-Ratten.

I. Leber nach normaler Diät.
II. Leber nach 4-monatl. Buttergelb-Behandlung.
III. Beginnendes Hepatom nach 4-monatl. Buttergelb-Behandlung.

Succino-Dehydrase in der normalen Leber von BD I-Ratten

Leber: BD I, Nr. 6, Aug. 52, Gew. 412 g. Leber 12 g.

Versuch I

Eingewogen 1,04 g Leber + 10 ml Phosphatpuffer.

Leber	1	1	0,5	0,5	0,25	0,25	
Puffer	1,25	1,25	1,75	1,75	2	2	
Succinat	0,25	0,25	0,25	0,25	0,25	0,25	
Metylenblau	0,5	0,5	0,5	0,5	0,5	0,5	
Entfärbung in . . .	3 $^1/_2$	3 $^1/_2$	10	10	>30	>30	Min.

Leber	1	1	0,5	0,5	0,4	0,4	
Puffer	1,25	1,25	1,75	1,75	1,85	1,85	
Succinat	0,25	0,25	0,25	0,25	0,25	0,25	
Metylenblau	0,5	0,5	0,5	0,5	0,5	0,5	
Entfärbung in . . .	4 $^1/_2$	4 $^1/_2$	9 $^1/_2$	9 $^1/_2$	12 $^1/_2$	12 $^1/_2$	Min.

Versuch II

Eingewogen 1,3 g Leber + 10 ml Phosphatpuffer.

Leber	0,75	0,75	0,5	0,5	0,3	0,3	
Puffer	1,50	1,5	1,75	1,75	1,95	1,95	
Succinat	0,25	0,25	0,25	0,25	0,25	0,25	
Metylenblau	0,5	0,5	0,5	0,5	0,5	0,5	
Entfärbung in . . .	4 $^1/_2$	4 $^1/_2$	9	9	15	15	Min.

Leber	1	1	0,5	0,5	
Puffer	1,5	1,5	2	2	
Succinat	—	—	—	—	
Metylenblau	0,5	0,5	0,5	0,5	
Entfärbung in . . .	6 $^1/_2$	6 $^1/_2$	30	15	Min.

Nach Dialyse: 2 ml Leber entfärbt in 11 Min., ohne Succinat über 40 Min. (40 % Entfärbung) 1 ml Leber in 23 Min.

Leber: BD I, Aug. 52, Gew. 300 g. Leber 14 g.

Succino-Dehydrase nach Buttergelb-Behandlung in der Leber von BD I-Ratten.

Versuch I

Eingewogen 1,03 g Leber (gelbe Tumoren mit unbedeutender Lebermänge). 7 + 10 ml Phosphatpuffer. (Tumorreichster Teil der Leber.)

Leber	1	1	0,5	0,5	0,4	0,4	
Puffer	1,25	1,25	1,75	1,75	1,85	1,85	
Succinat	0,25	0,25	0,25	0,25	0,25	0,25	
Metylenblau	0,5	0,5	0,5	0,5	0,5	0,5	
Entfärbung in . . .	14	14	—	30	45	45	Min.

Leber	1	1	0,5	0,5	
Puffer	1,5	1,5	2	2	
Succinat	—	—	—	—	
Metylenblau	0,5	0,5	0,5	0,5	
Entfärbung in . . .	—	30	>30	>30	Min.

Versuch II

Eingewogen 1,00 g Leber (ohne Tumor), Spitze der Leber + 10 ml Phosphatpuffer.

Leber	2	2	1	1	0,5	0,5	1	1	
Puffer	0,25	0,25	1,25	1,25	1,75	1,75	1,5	1,5	
Succinat	0,25	0,25	0,25	0,25	0,25	0,25	—	—	
Metylenblau	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	
Entfärbung in	4	4	11	12	20	20	20	20	Min.

Nach Dialyse: 2 ml Leber entfärbt in 30 Min., ohne Succinat über 40 Min. (50 % Entfärbung).

fliessendes Wasser dialysiert um den grössten Teil der organ-eigenen Substrate zu entfernen und dann teils als Leber-Suspension teils zur Extraktion mit Pufferlösungen verwendet.

Zu den Versuchen mit Citrico- und mit Lactico-Dehydrogenase wurden variierende Mengen von Cozymase bis zur vollständigen Aktivierung zugesetzt, welche unter der Leitung von Herrn FRITZ SCHLENK von Frln. INGA PETTERSSON nach der Vorschrift von FR. SCHLENK[1] dargestellt wurde.

Der Einfluss von Chinonimid hat uns interessiert in Zusammenhang mit der Frage nach dem Einfluss von Oxydationskatalysatoren auf die Tumorentwicklung. Andere hierhergehörende Versuche werden später mitgeteilt.

In dieser Mitteilung sei nur hingewiesen auf den erheblichen Unterschied zwischen der Aktivität Cozymase-bedingter Dehydrasen in der Leber normaler und Tumor-tragender Ratten.

B. Lebergewebe von normalen und mit Buttergelb behandelten Ratten

Zunächst wurden zu oben genannten Vergleich Tiere unseres norwegischen, weissen Rattenstammes verwendet; die mit diesen erhaltenen Ergebnisse sollen noch ergänzt und dann später mitgeteilt werden.

Die zu den nachstehend erwähnten Vorversuchen verwendeten gelben Ratten des Stammes BD I erhielt das Vitamin-Institut von Herrn Professor Dr. H. DRUCKREY, Freiburg-Breisgau, dem wir auch an dieser Stelle für alle Hülfe verbindlichst danken wollen. Diese Ratten von welchem das Institut nun die 3. Generation gezüchtet hat, werden verwendet zum Studium des Einflusses von Dimethyl-amino-azobenzol auf die Entwicklung von Hepatomen.

Die Ratten erhalten in diesem Institut eine Riboflavin- und Protein-arme Kost, welche in einer folgenden Mitteilung genauer beschrieben wird.

Die Versuchsmethodik war ähnlich der früher mit der Citrico- und Lactico-Dehydrase verwendeten. Cozymase-Zusatz war hier nicht erforderlich. Das Zerreiben des Leber-Materiales wurde bei diesen Versuchen wesentlich schneller mit einem Homogenisator, BÜHLER, Tübingen durchgeführt.

Die Ergebnisse einiger Vorversuche sind in den Tabellen S. 463 angegeben und in Fig. 3 zusammengefasst.

Stockholm, Inst. für organ.-chem. Forschung und Vitamin-Institut.

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[1] Vgl. F. SCHLENK u. EULER, Fortschr. d. Chemie org. Naturst. 1, 99; 1938.

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Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

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Starch degradation by the α-amylases

IV. Maltase action of saliva

By Karl Myrbäck and Ebba Willstaedt

With 3 figures in the text

In an earlier paper of this series (1) it was found that when large amounts of saliva were allowed to act upon so-called residual dextrins for long periods of time, maltose was first formed but disappeared gradually with the formation of glucose. It was concluded that maltose is present in saliva or at least in certain samples of saliva. Since this has been denied by certain investigators (2) we have found a reinvestigation necessary. We are quite aware that the amounts of maltase in saliva must be very small, but the question of its presence is of no little interest for the understanding of the amylase action on starch (glycogen) and its degradation products.

Experimental

Enzyme. Saliva was filtered through a layer of diatomaceous earth and stored under toluene at $+2^0$.

Substrates. Maltose of best commercial quality and a specimen of Lintner starch prepared in the usual way from potato starch.

Maltase action of saliva.

a) 1 g Maltose was dissolved in 10 ml saliva and stored under toluene at 40° for 4 days;

b) A sample of the same saliva was heated to 63° for 15 minutes and then used for a similar experiment.

1 ml of the solutions was evaporated to dryness, dissolved in 1 ml water and 2 ml suspension of baker's yeast was added. The carbon dioxide evolution was measured and the amounts of glucose and maltose present were calculated as described in earlier papers (3, 4). The fermentation experiments and some parallel experiments with known amounts of glucose and maltose are shown in fig. 1.

The fermentation curves in fig. 1 show that in experiment a) not less than about 50 percent of the maltose has been split to glucose. In experiment b) much less glucose (<5 percent) has been formed. Since the heated saliva was still strongly active on starch it can be concluded that maltase has been destroyed selectively to a certain extent.

That large amounts of glucose have been formed by the action of saliva on maltose is evident also from the paper chromatographical examination: Chromatograms Nos. 2 and 4 in fig. 2 belong to experiment a) and were carried out after 15 days

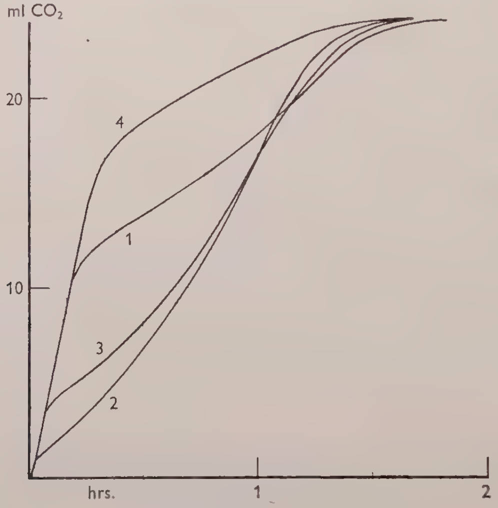

Fig. 1. Fermentation curves.

Curve 1: Experiment a.
2: Experiment b.
3: 20 mg glucose + 80 mg maltose.
4: 80 mg glucose + 20 mg maltose.

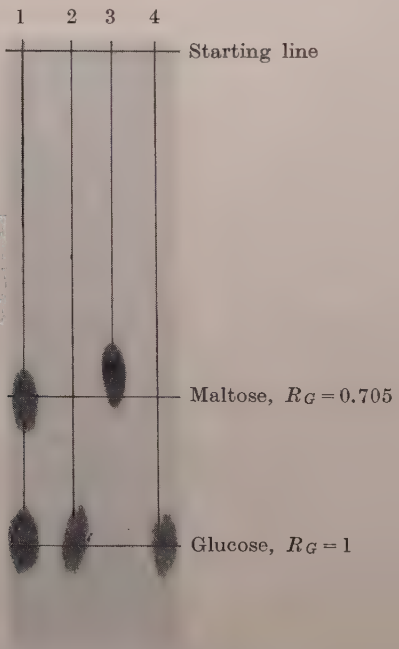
1
2
3
4
Starting line
Maltose, $R_G = 0.705$
Glucose, $R_G = 1$

Fig. 2. No. 1: Experiment b), 17 days.
Nos. 2 and 4: Experiment a), 15 days.
No. 3: Maltose.

Table 1.

Reaction time	Experiment											
	c)		d)		e)		f)		g)		h)	
	Reducing power %	% glucose	Reducing power %	% glucose	Reducing power %	% glucose	Reducing power %	% glucose	Reducing power %	% glucose	Reducing power %	% glucose
1 h			31.9				31.7				30.0	
20			38.3	3.2	52.1	2.5	38.8	4.5	50.0	0		1.2
45										0		2.5
70			44.8									
96					53.0	5		10.5		0		
120	55	8										
190			47.5	11.5					51	~2		7.5
216	58	15			54	6		12.5				
10 days				15.0								
15 »										~2		10.7
17 »								14.0				
27 »										~2		
30 »						6.5		14.0				11.3
50 »										5		13.8
53 »						10.0		18.5				

action of the enzyme. The hydrolysis to glucose is almost complete. No. 3 is a reference experiment with maltose alone. Chromatogram No. 1 belongs to experiment b) and was carried out after 17 days action of the enzyme. Obviously large amounts of glucose have been formed.

Action of heated enzyme on maltose and on starch. Comparative experiments were performed with saliva heated to different temperatures and with maltose and Lintner starch as substrate, respectively. This was done in order to establish if the formation of glucose from starch by saliva is parallel to the action of the enzyme on maltose. *A priori* it cannot be excluded that the enzyme may liberate glucose from dextrins even if it has no action on maltose.

c) Saliva heated to 61° for 15 minutes. 200 ml 2 percent maltose solution, 2 ml phosphate buffer ($pH = 6.9$) and 0.1 g NaCl were mixed and brought to 40°. 5 ml saliva was added and the mixture stored at this temperature under toluene. The reducing power was determined (Willstätter and Schudel method). Glucose was determined in fermentation tests with baker's yeast as described above.

d) Same experiment as c) but 2 percent Lintner starch solution instead of maltose.

e) and f). Same as c) and d) but saliva heated to 62°.

g) and h). Same as c) and d) but saliva heated to 63°.

The results are collected in table 1. The maltose values found in the fermentation experiments are not given in the table; after 20 hrs reaction time they exceeded 75 percent in all experiments with starch.

It appears from the table that the action of the enzyme on maltose is strongly reduced after heating of the solution above 60°. A solution heated to 63° for 15 minutes has almost no action on maltose. The glucose formation in the parallel

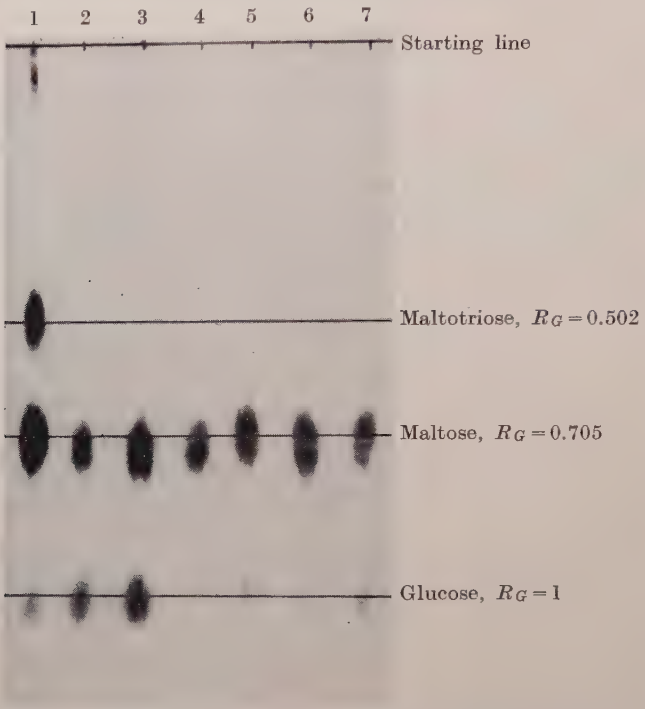

Fig. 3. No. 1: Experiment h), 24 hrs.
No. 2: Experiment h), 50 days.
No. 3: Glucose + maltose, 1 : 3.
No. 4: Experiment g), 48 days.
No. 5: Glucose + maltose, 1 : 24,
No. 6: Experiment g), 53 days.
No. 7: Glucose + maltose, 1 : 12.

experiments with starch as the substrate also decreases after heating of the enzyme. Obviously this must be the case if glucose is formed from maltose by the action of a contaminating maltase. However, if we compare the experiments with maltose and those with starch as substrates, and especially the experiments g) and h), it appears that considerably more glucose is formed from starch than from maltose. In agreement with earlier experiments (5) these results support the belief that salivary amylase may liberate glucose from other substrates than maltose.

The nature of the low-molecular reaction products in the experiments c)–h) was also investigated by means of paper chromatography. The results agreed well with those arrived at in the fermentation experiments. However, by paper chromatography separation of maltose and maltotriose is easy (6), whereas the fermentation method only yields the sum of both sugars. A few chromatograms are shown in fig. 3. Chromatogram No. 1 corresponds to experiment h) and a reaction time of 24 hrs. Small amounts of glucose are already present together with large amounts of maltose. Interesting is the presence of a strong spot having precisely the R_G-value corresponding to maltotriose. (The horizontal lines in the figures denote the R_G-values determined by us in a previous communication (6).) This agrees with the belief expressed earlier in papers from this laboratory that maltotriose is stable, or practically stable, toward salivary amylase (7). The same result was reported by WHELAN *et al.* (8).

Chromatogram No. 2 in fig. 2 corresponds to experiment h) and a reaction time of 50 days. The glucose spot is stronger but the maltotriose spot has vanished. This means either that maltotriose is hydrolyzed at a very low rate by the salivary amylase or that the enzyme preparation used in our experiment contains some other enzyme capable of attacking maltotriose.

Chromatograms Nos. 4 and 6 correspond to experiment g), reaction times 48 and 53 days, respectively. The glucose spots are rather weak. Chromatograms Nos. 3, 5, and 7 are reference experiments with maltose containing 25, 4, and 8 percent glucose, respectively. A comparison of the glucose spots in chromatograms Nos. 4–7 confirms the result of the fermentation experiments, *viz.* a formation of about 5 percent glucose in experiment g).

Summary

Saliva (or certain samples of saliva) contains traces of maltase, which may interfere in the determination of the end-products of starch degradation by salivary amylase.

Glucose is formed when saliva acts on starch paste. Part of this glucose may originate from maltose.

Small amounts of glucose are formed from starch in experiments with heated saliva, that has practically no action on maltose. It must be concluded that glucose is formed also from dextrins with more than two glucose units. However, this glucose formation may possibly be caused by an enzyme other than the salivary amylase proper or maltase.

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4. Myrbäck, K. and Willstaedt, E., Arkiv Kemi *4*, 523 (1952).
5. Myrbäck, K. and Svanborg, K., Arkiv Kemi *5*, 317 (1953).
6. Myrbäck, K. and Willstaedt, E., Arkiv Kemi *6*, 417 (1953).
7. Myrbäck, K. and Leissner, E., Arkiv Kemi Mineral. Geol. *A 18*, No. 18 (1943).
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Tryckt den 7 december 1953

Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

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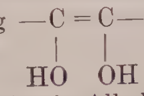
Vorstufen der Redukton-Bildung aus Hexosen

Von HANS VON EULER, HANS HASSELQUIST und GUNNAR HANSHOFF

Mit 5 Figuren im Text

Die hier mitzuteilenden Versuche schliessen sich an zwei unserer vorhergehenden Arbeiten an, von denen die *erste* die Aziditäten behandelt, bei welchen Reduktone das Maximum ihrer Stabilität gegen Tillmans' Reagens (TR) erreichen[1], während in der *zweiten*[2] die Beziehungen verglichen werden, welche einerseits zwischen Aldehydform und Halbazetalform der Glukose, andererseits zwischen der Laktonform der Askorbinsäure und ihrer offenen Ketoform bestehen.

Dass Dichlorphenol-indophenol (Tillmans-Reagens, TR) durch Glukose und durch andere Hexosen *nur* in alkalischer Lösung reduziert wird, ist in vorhergehenden Arbeiten schon betont worden; es blieb aber festzustellen, bei welcher Alkalinität, bei welchem pH, die Geschwindigkeit der TR-Entfärbung durch Glukose mit der durch definierte Reduktone erzeugten vergleichbar wird, bei welchem pH also die Atomgruppierung  $\underset{\mathrm{HO}}{-\mathrm{C}}=\underset{\mathrm{OH}}{\mathrm{C}-}$ in Reaktion zu treten beginnt.

Was die hierbei mit Alkali eintretende Salzbildung betrifft, so ist dieselbe für reine (nicht umgelagerte) Glukose berechenbar aus der schon vor langer Zeit gemessenen elektrolytischen Dissoziationskonstanten $K_a = 10^{-13}$. Sobald aber in einem Teil der Glukose-Moleküle die Endiol-Gruppe gebildet worden ist, haben wir für diesen als Endiol vorliegenden Teil der Glukose eine Dissoziationskonstante anzunehmen, welche etwa derjenigen des Triose-Reduktons entspricht, also der Grössenordnung 10^{-5} gleichkommt. Voraussetzung für die Richtigkeit einer solchen Berechnung ist allerdings, dass während der Salzbildung keine Konstitutionsveränderung an der zu messenden Substanz eintritt.

I. Geschwindigkeit der TR-Reduktion in alkalischer Glukose-Lösung

Die Entfärbung des TR erfolgt bei einer Gruppe von Reduktonen, z. B. bei Triose-Redukton und bei Askorbinsäure, sehr schnell, meist im Verlauf von Sekunden; bei einer anderen Gruppe von Reduktonen ist die Geschwindigkeit der TR-Entfärbung in saurer Lösung sehr gering und nimmt mit steigender Alkalinität stetig zu.[3]

Die bei der Einwirkung von Alkali auf einfache Zuckerarten eintretenden Vorgänge sind in unserem Arbeitskreis teils in Zusammenhang mit der Kinetik der Mutarota-

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[1] EULER, H. HASSELQUIST u. O. CEDER, Liebigs Ann. Chem. 581, 189; 1953.

[2] EULER, H. HASSELQUIST u. G. HANSHOFF, Z. Naturforsch. 8 b, im Druck.

[3] EULER, H. HASSELQUIST u. O. CEDER, l. c. — Siehe auch F. PETUELY u. U. KÜNSBERG, Monatshefte Chemie 83, 80; 1952.

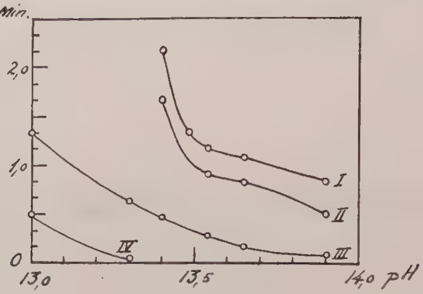

Fig. 1.

Entfärbungs-Geschwindigkeit in alkal. Zuckerlös.

Kurve 1: Glukose	Kurve 3: Fruktose
,, 2: Galaktose	,, 4: Dioxyazeton

tion verfolgt worden[4], teils in Zusammenhang mit den aus Aldo- und Keto-Hexosen enstehenden Reduktonen[5].

Die erste quantitative Untersuchung, wie sich die Fähigkeit von Reduktonen, das Tillmans-Reagens zu reduzieren, mit der Azidität der Reaktionsmischung ändert, wurden von EULER, HASSELQUIST und CEDER[3] an Triose-Redukton und an Askorbinsäure ausgeführt. Sie ergab sehr scharfe Minima der Reaktionsgeschwindigkeit bei pH 8,5 bzw. ~ 10 (etwas abhängig von der Konzentration des Substrates).

Die Lage dieser Minima im alkalischen Gebiet ist natürlich auch durch die andere Reaktionskomponente, TR, beeinflusst.

Die folgenden, an *Hexosen* ausgeführten Versuche sind, abweichend von unseren eben erwähnten, an Reduktonen angestellten Bestimmungen, folgendermaassen angeordnet worden:

Die Mischung von

1 ml einer 1 molaren Hexose-Lösung + 0,2 ml einer 0,01 norm. TR-Lösung wurde bei 20° unter Stickstoff schnell mit 0,8 ml Natronlauge von solcher Normalität versetzt, dass die 2 ml betragende Reaktionsmischung die in der Fig. 1 angegebenen pH-Werte erreichte.

Analoge Versuche wurden mit $Ba(OH)_2$ statt mit NaOH angestellt, ergaben aber keine wesentlich abweichenden Resultate. Die Endiolat-Bildung war also die gleiche wie mit NaOH.

Wie Fig. 1 zeigt, handelt es sich bei diesen Versuchen um Reaktionszeiten, welche 2 Minuten nicht übersteigen; man kann annehmen, dass in den untersuchten Lösungen in 2 Minuten noch kein Triose-Redukton gebildet worden ist. Die ermittelten Reaktionszeiten liefern einen Anhaltspunkt für den Gehalt der verwendeten Hexose an Endiol bzw. an Endiolat. Die Glukose in dem durch Fig. 1, Kurve I dargestellten Versuch dürfte demnach bei pH 13,5 weniger als 1 % in der Endiol-Form enthalten[6], und das angenähert Gleiche gilt von der Galaktose in Versuch Fig. 1, Kurve II.

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[4] EULER, A. ÖLANDER u. E. RUDBERG, Z. anorg. u. allgem. Chem. 145, 58; 1925.

[5] EULER u. H. HASSELQUIST, Die Reduktone. Stuttgart, Enke; 1950.

[6] Es bestätigt sich also, dass unter den Umständen, unter welchen wir früher die Mutarotation der Glukose gemessen haben[4] kein Endiolat vorhanden war, und es liegt kein Grund vor, zur Annahme, dass der Halbazetal-Ring der Glukose im pH-Bereich 7–12 geöffnet wird. Wir stimmen

In der parallel hierzu untersuchten Fruktose-Lösung (Kurve III) ist offenbar der Endiol-(Endiolat-)Gehalt wesentlich (etwa 3–5 mal) grösser.

Der Unterschied der Kurven der Fig. 1 gegenüber den früher[1] mit Triose-Redukton und mit Askorbinsäure erhaltenen legt wieder die Frage nahe, welche Elektronen-Verteilung die Stabilität der Endiol-Gruppe in saurer Lösung bedingt. Die Antwort, dass die Endiolform einer Substanz durch eine benachbarte Keto-Gruppe stabilisiert wird, enthält natürlich keine wirkliche Lösung des Problems.

II. Absorption alkalischer Hexose-Lösungen im UV

Material: Glukose, puriss. zweimal aus Alkohol umkrystallisiert
Fruktose, puriss. krystallisiert Schering-Kahlbaum
Galaktose, puriss. Pfanstiel, Central Scientific Co., Chicago.

Alle photoelektrischen Ablesungen bei Zimmertemperatur 20° im Beckman-Quarz-Spektrophotometer Modell 2500, California.

Die hochgereinigte Glukose zeigt in neutraler ($pH = 6$) wässriger Lösung ganz geringe kontinuierliche Absorption ohne Maxima. Siehe hierzu die Arbeiten von V. Henri u. Schou[7] und von H. H. Schubach u. Mitarb.[8].

An Triose-Redukton erhielt H. Hellström[9]:

bei der Azidität	die Maxima
pH 2–4	268 mμ
pH 6–10	287 mμ

Für Lösungen, in denen sowohl die Hexosen als auch NaOH 0,5 normal waren, wurden die Maxima gefunden:

Marchlewski u. Mitarb.[10]	Glukose	267,6 und 311,2 mμ
	Galaktose	268,7
Hanshoff	Glukose, pH 12,8	310,0 mμ

Der Extinktionswert von Marchlewski 270 mμ ist nicht konstant. Der Extinktionswert 310–320 mμ nahm bei den Messungen von Hanshoff während der ersten 15 Minuten schnell, dann recht langsam zu. Dagegen verblieb der Verbrauch an TR auch nach 15 Minuten unverändert. Die Extinktion der alkalischen Glukose-Lösung kann nicht von Glukose-Endiol herrühren, da dessen Verbrauch unverändert weiter geht, während die Absorption (im Vergleich zum TR-Verbrauch) konstant bleibt. Wir entnehmen unseren Messungen, dass die beobachtete Extinktion identisch mit derjenigen des Triose-Reduktons ist.

Galaktose. Zum Vergleich mit den Messungen von Marchlewski u. Mitarb. (l. c.) wurde eine Reaktionsmischung der gleichen Teile der 1 molaren Lösungen von Zucker und Alkali bei 20° untersucht. In Fig. 2 zeigt sich die Verschiebung des Absorptions-Maximums während einer Reaktionszeit von 3 Tagen, die einer Bildung von Triose-Redukton entspricht.

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in dieser Hinsicht mit F. Petuely (Vortrag Innsbruck, März 1953, Ref. Angew. Chemie 65, 268; 1953) durchaus überein.

[7] V. Henri u. S. A. Schou, Z. physiol. Chem. 186, 148; 1929.

[8] H. H. Schlubach u. Mitarb., Z. physiol. Chem. 174, 295; 1928.

[9] Siehe Euler u. C. Martius, Lieb. Ann. Chem. 505, 80; 1933.

[10] W. Gabrielski u. L. Marchlewski, Biochem. Zs. 250, 385; 1932. — 261, 393; 1933.

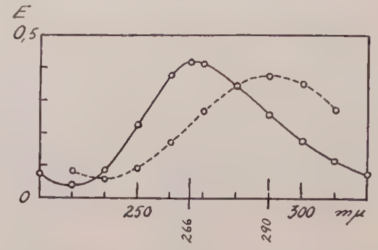

Fig. 2.

Galaktose 0,5 molar NaOH 0,5 norm. Gemessen 3 Tage nach der Mischung.
Konz. bei der Messung: $1{,}61 \cdot 10^{-3}$ mol. Galaktose.
Konz. des mit TR titrierten Reduktons: $1{,}66 \cdot 10^{-5}$.
Konz. des Reduktons, berechnet aus der Extinktion: $1{,}82 \cdot 10^{-5}$.
Kurve ——— pH 1 Kurve - - - - - pH 11.

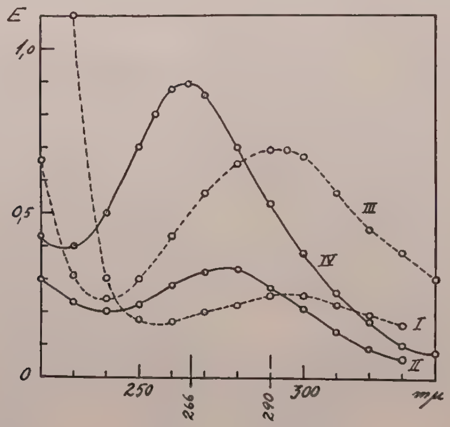

Fig. 3. Fruktose.

Fruktose. Die Versuche werden durch die Kurven der Fig. 3 dargestellt.

Kurve I: Konz. 0,05 mol., nach 30 Minuten pH = 11
,, II: Konz. 0,05 mol., ,, ,, ,, pH = 1
,, III: Konz. 0.01 mol., ,, 24 Stunden pH = 11
,, IV: Konz. 0,01 mol., ,, 24 Stunden pH = 1

5 ml der 0,5 molaren angesäuerten Lösung verbrauchten 1,7 ml einer 0,01 norm. TR-Lösung

Die in Kurve IV dargestellte Lösung war $3{,}4 \cdot 10^{-5}$ molar in Bezug auf Redukton, gemessen durch Titration mittels TR. Die Absorption entspricht $4 \cdot 10^{-5}$ molar. Redukton. Die Übereinstimmung zwischen der chemischen und der optischen Bestimmungsmethode ist also befriedigend.

Wie die Figur 3 zeigt, verschiebt sich das nach 24 Stunden eintretende Absorptions-Maximum zwischen pH 11 und pH 1 von 266 mμ bis 290 mμ.

Cu-Katalyse der Redukton-Bildung aus Glukose

Über die Kinetik der Bildung von Triose-Redukton aus Glukose ist bisher wenig bekannt geworden. Es handelt sich vermutlich um mehrere aufeinanderfolgende

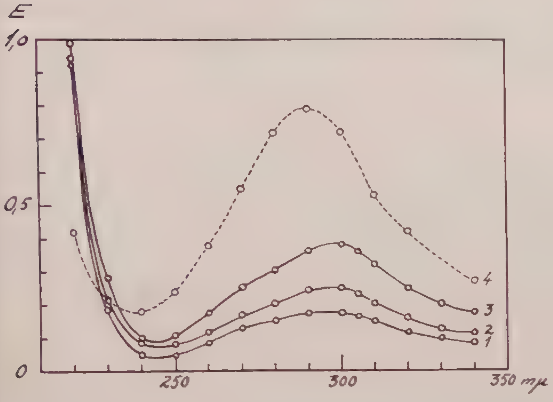

Fig. 4.

10 ml 1-mol. Glucoselös. + 10 ml 1-norm. NaOH

Kurve 1: Ohne Cu-Zusatz
,, 2: 10 mg $CuAc_2$/100 ml } nach 24 Stunden. pH 11,7.
,, 3: 50 mg $CuAc_2$/100 ml
,, 4: 50 mg $CuAc_2$/100 ml nach 73 Stunden. pH 11,1.

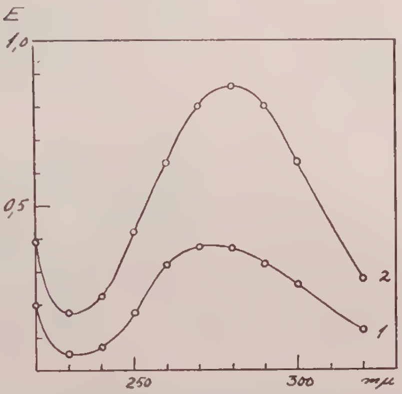

Fig. 5.

Glukose 1 molar + NaOH 1 molar.

Kurve 1: Ohne Cu-Zusats
,, 2: 10 mg $CuAc_2$/100 ml.
Nach 1 Stunde.

Reaktionen, nämlich die Hydrolyse des Halbazetalringes der Glukose, die Bildung eines Endiolates und schliesslich die Trennung der C_3–C_4-Bindung. Diese Reaktionsfolge wird durch sehr kleine Mengen von Cu-Ionen katalysiert, und es bleibt noch aufzuklären, welche der genannten Reaktionstypen Cu-empfindlich ist.

Fig. 4 zeigt die im Verlauf von 1 Stunde eintretende Erhöhung des Absorptions-Maximums bei 280 mμ um etwa 100 %. Es geht aber aus der Fig. 5 hervor, dass nach dieser Zeit die katalytische Wirkung des Kupfersalzes nicht mehr zunimmt — vgl. die Kurven 1 und 2 — sondern dass die Höhen der Absorptions-Maximen angenähert gleich werden.

Wir kommen in einer folgenden Mitteilung auf die hier behandelten Umwandlungen der Hexosen vom enzymatischen Standpunkt aus zurück, insbesondere auf die Reaktionen des Blutzuckers bei Diabetikern.

ZUSAMMENFASSUNG

Im Gegensatz zu den das Tillmans-Reagens auch in saurer Lösung reduzierenden Reduktonen beginnt bei Glukose und anderen Aldohexosen in alkalischer Lösung die TR-Redukton erst bei Alkalinitäten von pH 12. Damit wird die Reduktionsfähigkeit von Fruktose quantitativ verglichen.

In mässig starken NaOH-Lösungen (0,1–1,0 norm.) zeigen Hexosen nach kurzer Zeit Absorptions-Maxima, die zum Teil von der Bildung von Triose-Redukton herrühren.

Frühere Untersuchungen über die Mutarotation der Glukose werden hierdurch nicht berührt.

Stockholm, Universität, Institut für organ.-chemische Forschung.

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Uppsala 1953. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 6 nr 45

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On the occurrence of picolinic acid betaine in sea urchin embryos

By TORE HULTIN, SVEN LINDVALL and KERSTIN GUSTAFSSON

With 2 figures in the text

The occurrence in certain marine Invertebrates of the methyl betaine of picolinic acid (homarine) was demonstrated several years ago by HOPPE-SEYLER (2). He was able to identify with this substance a base, $C_7H_7NO_2$, which had previously been prepared from the sea urchin *Arbacia lixula* (L.) by HOLZ, KUTSCHER and THIELMANN (1), and which had been considered as trigonelline by these authors.

In connection with a study of the metabolic background of sea urchin development our attention was directed towards a quaternary ammonium base, which is soluble in water and alcohol and characterized by a strong light absorption in the ultraviolet region. This base was isolated and found to be picolinic acid betaine. It seemed possible that this substance might have some significant metabolic function in the morphogenesis, e.g., by taking part in methylation reactions. In this connection, the characteristic ultraviolet absorption of the substance (Figures 1, 2) was utilized to study its occurrence in the eggs and embryos of a number of sea urchin species at different stages of development. A preliminary study of the incorporation of $N^{15}H_4Cl$ in the picolinic acid betaine of *Paracentrotus lividus* (LM) embryos was also carried out.

1. Ultraviolet absorption of picolinic acid betaine and related compounds

Picolinic acid and picolinic acid methyl betaine (homarine) were synthesized from α-picoline. Trigonelline was synthesized from commercial nicotinic acid by methylation. The ultraviolet absorption spectra of these compounds in 0.05 N HCl, 0.05 N NaOH, and 96 % ethanol are shown in Figures 1–2. The introduction of methyl groups into the picolinic acid and nicotinic acid molecules displaced the absorption maxima of both substances in the direction of higher wavelengths. This change was particularly pronounced in the case of picolinic acid. In water solutions, picolinic acid betaine showed an absorption peak at 2720–2730 Å, while trigonelline had a maximum at 2640 Å. In 96 % ethanol, the absorption peak of picolinic acid betaine shifted to 2780 Å in a very characteristic way, while the maximum of trigonelline remained very nearly the same as in water (2650 Å).

2. Isolation of picolinic acid betaine from eggs and embryos of sea urchins

Lyophilized sea urchin eggs and embryos at different stages of development were finely powdered in a mortar and thoroughly extracted with water. The extracts were deproteinized by addition of 10 % trichloroacetic acid (TCA), and afterwards treated

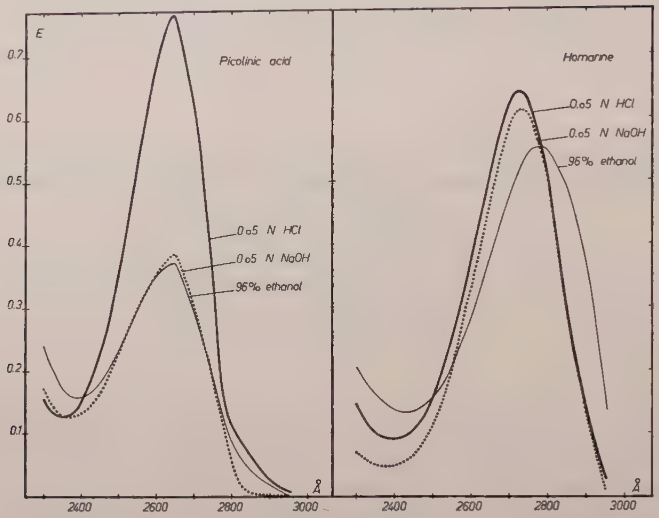

Fig. 1. Ultraviolet absorption curves of 0.0001 M picolinic acid and picolinic acid methyl betaine (homarine) in 0.05 N HCl, 0.05 N NaOH, and 96 % ethanol.

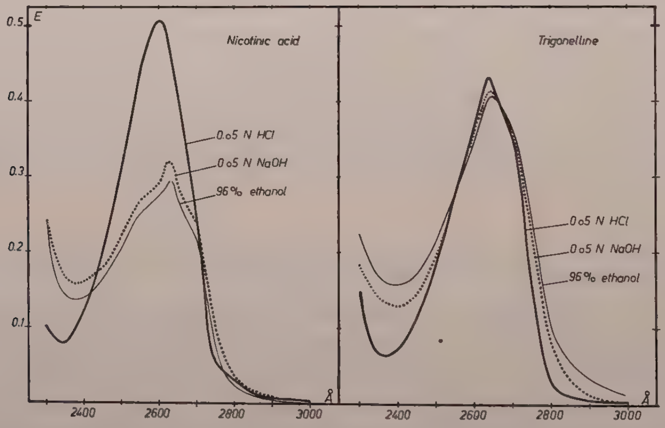

Fig. 2. Ultraviolet absorption curves of 0.0001 M nicotinic acid and nicotinic acid methyl betaine (trigonelline) in 0.05 N HCl, 0.05 N NaOH, and 96 % ethanol.

with ether in a liquid extractor in order to remove the TCA. The solutions were concentrated in vacuo and added to columns of the kation exchanger Dowex 50. Increasing concentrations of HCl (1–4 N) were used as solvent. The ultraviolet absorption of the eluates was measured in a Beckmann spectrophotometer. The picolinic acid betaine fraction appeared after the hypoxanthine fraction, and between the leucine and lysine fractions.

The central part of the picolinic acid betaine fraction was evaporated in vacuo and the chloride (which is more soluble in alcohol than the chloride of glycine betaine) taken up in a small volume of ethanol. It was then precipitated with $HgCl_2$

and the precipitate decomposed with H_2S. The solution was concentrated to a small volume in vacuo and made slightly alkaline by addition of Na_2CO_3. After addition of I_2–KI reagent (5) in slight excess, the mixture was kept over night at 0° C. Under these conditions traces of choline would be quantitatively precipitated (together with a minor portion of the picolinic acid betaine). The precipitate was removed and the filtrate diluted. The main part of the picolinic acid betaine was then precipitated by the addition of a solution of iodine in hydroiodic acid. The periodide of the picolinic acid betaine precipitated at room temperature as a heavy oil. It was carefully washed in water, dissolved in ethanol, and repeatedly evaporated with ethanol and HCl in vacuo. The resulting chloride was dissolved in a small volume of alcohol, and precipitated with ether. The substance was converted into the chloroaurate, and recrystallized from HCl.

Paracentrotus embryos at different stages of development were treated for 5 hours with $N^{15}H_4Cl$ (61 % N^{15} excess). Immediately after the isotope treatment, the embryos were washed and lyophilized. The preparation of this isotopic sea urchin material has previously been described in detail (3, 4). From this N^{15}-labeled material, picolinic acid was isolated according to the method described. It was found, however, that the rate of utilization of the isotope for the formation of picolinic acid betaine was very small. Even at the gastrula stage, the isotope contents did not exceed 0.02 % N^{15} excess.

By means of the same chromatographic method, picolinic acid betaine was also found to occur in the eggs or embryos of *Arbacia lixula* (L.), *Sphaerechinus granularis* (LM.), *Psammechinus microtuberculatus* (Blainv.) and *Echinus esculentus* (L.). The substance could not be demonstrated in the eggs of *Strongylocentrotus droebachiensis* (O. F. Müller), *Psammechinus miliaris* (Gmelin), *Echinocardium cordatum* (Penn.), *E. flavescens* (O. F. Müller) or *Asterias rubens* (L.). By measuring the ultraviolet absorption of the chromatographic fractions, unfertilized Paracentrotus eggs were found to contain about 10 mg of picolinic acid betaine per g dry weight, and Echinus eggs approximately 2.5 mg.

Samples of lyophilized, normal and lithium-treated Paracentrotus embryos at 11 stages of development, covering the whole range between fertilization and pluteus stage, were prepared. The samples were finely powdered, and 20 mg of each sample were extracted for 2 days at room temperature with 10 ml of 96 % ethanol. The ultraviolet absorption of the extracts was measured, and the absorption maxima at 2780 Å were recalculated on the bases of equal phosphorus contents in the samples. No significant alterations were observed during the course of normal or Li-vegetalized development, nor were changes observed in the shape of the absorption curves, which could indicate a considerable demethylation of picolinic acid betaine to picolinic acid.

SUMMARY

Picolinic acid methyl betaine (homarine) was found to occur in substantial amounts in the eggs and embryos of a number of sea urchin species. In several other species, however, the substance could not be demonstrated. It is distinguished by a characteristic light absorption in the ultraviolet region. $N^{15}H_4Cl$ is not readily incorporated into picolinic acid betaine during the development of *Paracentrotus lividus* embryos. The contents of the betaine in *Paracentrotus* embryos did not change significantly during the course of normal development or under the influence of LiCl.

The sea urchin material was collected at the Zoological Stations of Naples and Kristineberg. The authors are much indebted to the Authorities of the Stations for material and laboratory facilities.

The Wenner-Gren Institute for Experimental Biology, Stockholm.

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Electrophoretic studies of cobalamins II

By L.-E. ERICSON and H. NIHLÉN

With 1 figure in the text

In a previous paper on the electrophoretic properties of cobalamins [1] it was shown that vitamin B_{12} (cyanocobalamin) possesses both acidic and basic groups, giving the compound an isoelectric point near pH 1.9. It was stated that both the acidic and the basic groups of cyanocobalamin are weak. The acidic character of the vitamin B_{12}-cyanide complex (di- or tri-cyanocobalamin) was also demonstrated.

The hydroxo- and aquocobalamins (vitamin B_{12a} [2], vitamin B_{12b} [3, 4, 5]) have now been investigated in the same manner and the results obtained are reported in the present paper.

Experimental

Aquocobalamin was prepared by illumination of cyanocobalamin. The cyanocobalamin (vitamin B_{12}) used was supplied by The Distillers Company, Speke, Liverpool, England. As some vitamin B_{12}-preparations contain not only cyanocobalamin but many other vitamin B_{12}-factors with different electrophoretic properties [6, 7], the vitamin B_{12} used in the present study was submitted to paper ionophoretic and paper chromatographic studies before use. It was then observed that the vitamin B_{12}-preparation mentioned contained mainly cyanocobalamin while only traces of other factors (chiefly compounds with ionophoretic and chromatographic properties similar to those of factor C [8, 9] and vitamin B_{12s} [10], i.e. compounds with acidic features) could be detected. The concentration of vitamin B_{12}-factors other than cyanocobalamin was too low to interfere with the results of this investigation.

The cyanocobalamin (43 mg) was dissolved in 200 ml of glass-distilled water and the pH adjusted to 4.2 with hydrochloric acid. Nitrogen was bubbled through the solution which was exposed to daylight. The conversion of cyanocobalamin to aquocobalamin was followed spectrophotometrically. When the spectrum of the solution showed the characteristic maxima of aquocobalamin (3525 and 5280 Å) the solution was evaporated in vacuo to a volume of a few ml. The aquocobalamin was precipitated by addition of acetone and recrystallized once. The crystalline material was dried in vacuo at about 35° C.

The electrophoresis apparatus was of the Tiselius-Svensson type [11]. The experiments were run in preparative cells at 0° C. Buffer solutions having an ionic strenght of 0.1 and covering a pH-range from 2.0 to 12.2 were used. Weighed amounts

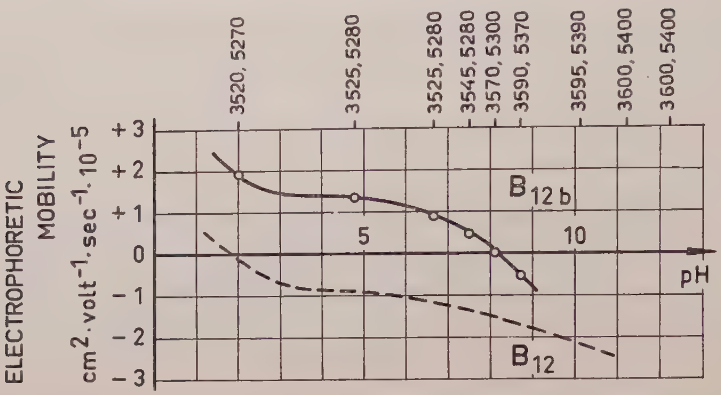

Fig. 1. Electrophoretic mobilities of aquo- and hydroxocobalamins at different pH-values. The dotted line shows the electrophoretic mobility of cyanocobalamin [1]. The figures above the curves show the shift in the spectrum maxima. Cyanocobalamin showed maxima at 3615 and 5520 Å in the Beckman Model DU spectrophotometer used.

of aquocobalamin were dissolved in the buffers to give solutions containing about 100 µg cobalamin/ml. These solutions also contained 2% glucose. The electrophoresis was carried out in a glucose gradient as described previously [1]. A diffusion time varying from three to five hours was used. The cells were continuously illuminated during the electrophoresis experiment to prevent conversion of the aquo- or hydroxocobalamin to cyanocobalamin, in case traces of cyanide due to impurities in the reagents used should be present in the buffer solutions (pro analysi chemicals were used throughout). The cobalamin content of the different chambers was determined spectrophotometrically. The spectra in the neighbourhood of the maxima for the aquo- and hydroxo-complexes was measured for every cobalamin determination to make certain that neither conversion nor destruction of the cobalamins had taken place.

Results

The curve obtained by plotting the electrophoretic mobility of the aquo- or hydroxocabalamin against the pH of the buffer solution is shown in Fig. 1. The corresponding curve for cyanocobalamin [1] is also given in this figure (the dotted line). The curve obtained from the data of the present investigation passes through the pH-axis at pH 8.1. The cobalamin existing below this pH-value moved towards the cathode, i.e. it was positively charged. The cobalamin existing above pH 8.1 was negatively charged. A bathochromic shift in the spectrum was observed as the cobalamin changed from being positively to becoming negatively charged (see Figure 1).

Electrophoresis experiments carried out at pH-values higher than 10.1 did not indicate that the acidic properties of the cobalamin existing in this pH-region should tend to equal those of cyanocobalamin. At pH 10.1 the cobalamin was still acidic and showed a definite electrophoretic mobility of $-0.85 \cdot 10^{-5}$ cm^{2}V^{-1}sec^{-1}. This value is, however, lower than would be expected from the curve showing the mobility values obtained at lower pH-values. At still higher pH-values (11.2 and 12.2), the determination of the cobalamin content of the two upper chambers indicated a migration towards the anode, whereas the cobalamin content of the two lower chambers indicated a migration towards the cathode. This phenomenon will be discussed later.

Aquocobalamin was dissolved in a sodium sulphite buffer and the pH adjusted to 10.4. The solution showed light absorption maxima at 3640 and 5150 Å which have been reported to be the characteristic maxima of sulphito-cobalamin [12]. Electrophoresis in a sulphite buffer (pH 10.4) of the cobalamin having these maxima showed that the cobalamin was only slightly acidic in nature. Its mobility value was $-0.75 \cdot 10^{-5} \mathrm{cm}^2 \mathrm{V}^{-1} \mathrm{sec}^{-1}$.

Discussion

Several authors have observed that the spectrum of vitamin B_{12a} [2] or vitamin B_{12b} [3, 4, 5] undergoes a bathochromic shift [4, 13–15]. For this reason and because of the basic properties of vitamin B_{12a} and B_{12b} [5, 12, 16–19], which have been demonstrated also by means of ionophoresis on paper [6, 9], the existence of an equilibrium between an aquo-form (aquocobalamin) and a hydroxoform (hydroxocobalamin) has been proposed [15, 18–20]. Using the symbols for cobalamins introduced by Buhs *et al.* [18] this equilibrium may be written:

$$\underset{\mathrm{I}}{\left[\begin{array}{l} --\mathrm{H_2O} \\ \mathrm{Co}^{+++} \\ \mathrm{O\,O\,O} \end{array}\right]^{+}} + \mathrm{OH}^{-} \rightleftarrows \underset{\mathrm{II}}{\left[\begin{array}{l} --\mathrm{OH}^{-} \\ \mathrm{Co}^{+++} \\ \mathrm{O\,O\,O} \end{array}\right]} + \mathrm{H_2O}$$

In this equation I represents the aquocobalamin and II the hydroxocobalamin. The electrophoretic experiments described in this paper provide evidence for the existence of this equilibrium between aquo- and hydroxocobalamins. The aquo-form dominates below and the hydroxo-form above pH 8.1. Consequently the greatest changes in the spectrum occurs near this pH-value (see Fig. 1). Parallel to the replacement of the H_2O-group of aquocobalamin by an OH^--group the spectrum of the product formed approaches that of cyanocobalamin. The fact that hydroxocobalamin migrates towards the anode at pH-values higher than 8.1 (i.e. 8.7 and 10.1) as does cyanocobalamin [1] again demonstrates the existence of acidic group(s) in the cobalamin molecule.

At high pH-values, however, a new reaction appears to take place which tends to produce a cobalamin (or some other compound) with more basic properties. The confusing phenomenon observed when the electrophoresis experiments were run at pH 11.2 and 12.2 (see Results) is not fully understood. The spectrum of the solutions of the different chambers closely resembled that of hydroxocobalamin which makes it unlikely that a destruction of the cobalamin had taken place. The more basic properties of a cobalamin existing in this pH-region could be explained on the assumption of the formation of a "di-cobalamin" in which two cobalamin molecules (K) have one OH^--group in common ($[K–OH–K]^+$). A compound of this type has been considered by Cooley *et al.* [21] to account for some differences between vitamin B_{12a} and vitamin B_{12b} observed by them. It is of interest to notice here that the "sulphitocobalamin" showed a mobility value at pH 10.4, lower than that of hydroxocobalamin at pH 10.1. We rather expected sulphitocobalamin to possess acidic features similar to those of the vitamin B_{12}-cyanide complex (di- or tricyanocobalamin) [1]. However, decomposition of sulphitocobalamin might take place under the conditions employed, as was in fact indicated by slight alterations in the spectrum.

It has been shown previously that the cyanocobalamin has an isoelectric point near 1.9 [1]. The derivate of the mobility-pH curve of cyanocobalamin has a maximum at this pH-value. The observation that the mobility-pH curve of aquocobalamin tends to show an increase in the derivate near pH 1.9 is of interest. A comparison of the curves for aquocobalamin and cyanocobalamin also shows that an increase of one unit charge causes an increase in the mobility value of about 2-2.2 $cm^2V^{-1}sec^{-1}$. The difference in mobility between the vitamin B_{12}-cyanide complex and cyanocobalamin is greater (at pH 10.55) i.e. about 3 $cm^2V^{-1}sec^{-1}$ [1]. It is therefore logical to assume that the vitamin B_{12}-cyanide complex is formed from cyanocobalamin by the addition of more than one CN^--group per molecule cyanocobalamin. This is in agreement with the findings of CONN *et al.* [22].

Vitamin B_{12a} [2] has been reported to have a pK_a of 6.9 by some authors [18] whereas others [19] have obtained a pK_a of 7.5 (the last value refers to vitamin B_{12b} [5, 19]). These pK_a-values, together with the fact that no net migration of cobalamin takes place at pH 8.1, gives pK_b-values of 4.7 and 5.3 respectively.

SUMMARY

Electrophoretic studies on aquocobalamin have demonstrated the existence of an equilibrium between this compound and hydroxocobalamin. The aquocobalamin dominates in solutions having pH-values below 8.1. The hydroxocobalamin, which shows acidic properties seems to be stable only up to approximately pH 10 at which new reactions appear to take place. It has been suggested that at higher pH-values the vitamin B_{12}-cyanide complex contains more than two cyanide radicals per molecule.

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Enzymatical and chemical studies on factors related to folic and folinic acid

By A. Bolinder, E. Widoff and L.-E. Ericson

With 4 figures in the text

The discovery of a new growth factor for *Leuconostoc citrovorum* 8081—the citrovorum factor [1] or folinic acid [2, 3]—which is closely related to folic acid [4, 5] has renewed the interest in the role of the folic acid group of factors in the nutrition of microorganisms and higher animals. The citrovorum factor can replace folic acid as a growth factor for *Streptococcus faecalis* [3, 6–10] and *Lactobacillus casei* [3, 6, 9, 10] and can also reverse the toxic effect of folic acid antagonists for these microorganisms [3, 10, 11]. The existence in natural material of microbiologically active higher conjugates of folinic acid has been proposed [12–20]. It has also been observed that folinic acid occurs in natural material in bound and inactive forms in a way similar to that of folic acid [9, 14–17]. Enzyme preparations effective in releasing folic acid from its bound forms have been found to be effective also in the liberation of factors active towards *L. citrovorum* [8, 9, 12–20].

In the present paper further studies on the enzyme systems acting upon the growth factors related to folic acid and folinic acid will be described. The enzyme systems used were those of chicken pancreas and hog kidney homogenates. Bacto yeast extract and synthetically prepared higher conjugates of folinic acid have been used as substrates. The products of the enzymatic action on these substrates have been investigated both quantitatively and qualitatively (by means of bioautographic methods) using the three test organisms *S. faecalis*, *Lb. casei* and *L. citrovorum*. Studies on the chemical degradation of some growth factors for these microorganisms have also been undertaken.

Experimental

Microbiological assays.—The microbiological activity of the various growth factors and extracts was determined by the agar cup plate method using the following test organisms: *Streptococcus faecalis* (ATCC 8043), *Lactobacillus casei* (ATCC 7469) and *Leuconostoc citrovorum* (ATCC 8081).

The test organisms were maintained on Bacto micro assay culture agar (Difco) and transferred every two or three weeks, when not frequently used. The cultures after incubation overnight at 37° C were stored in the refrigerator. Subcultures were made in centrifuge tubes filled with 5 ml portions of Bacto micro inoculum broth (Difco). The subcultures were incubated at 37° C for 12 h. In some instances 12 h incubation was not enough for *Lb. casei.* However, sufficiently rapid growth of *Lb. casei* occurred when the subcultures were prepared from a fresh stock culture.

For preparation of inocula the subcultures were centrifuged, washed twice with 0.9% sodium chloride solution, resuspended and diluted in sodium chloride solution. Dilutions were adjusted to a suitable turbidity with a Coleman Model 11 Universal spectrophotometer at 5000 Å. For *S. faecalis* an inoculum with a galvanometer reading of 50 was used and for *Lb. casei* and *L. citrovorum* a galvanometer reading of 70. The inocula did not remain in saline longer than one hour.

Portions of agar media (150 ml) were sterilized for 10 minutes at 110° C and then placed in a 50° C water thermostat for 1 h. Each portion was then mixed with 5 ml of inocolum and poured into the plate. The cup plates, which consisted of rectangular dishes, 17 × 39 cm, were filled with 150 ml agar medium. In the solid medium 60–100 cups, 6 mm in diameter, were bored. After pipetting the standard and test solutions (0.03 ml) into the cups, the plates were incubated at 37° C overnight. The diameter of the growth zone of each cup was calculated from the mean value of two diameters measured at right angles. The potency of the various factors and extracts were evaluated from the log dose-growth zone diameter line obtained from appropriate standard solutions.

For *S. faecalis* the Bacto folic acid assay medium (Difco) supplemented with 1.8% Bacto agar (Difco) was used.

The media for *Lb. casei* and *L. citrovorum* were similar in composition. The common part of the basal media for the two test organisms had the following composition per litre medium: Bacto-vitamin free casamino acids 5.0 g, DL-asparagine 200 mg, DL-alanine, L-cystine, cysteine hydrochloride and DL-tryptophane 100 mg each, adenine sulfate, guanine hydrochloride, uracil and xanthine 10 mg each, thiamine hydrochloride, riboflavin and calcium pantothenate 0.5 mg each, niacin 1 mg, pyridoxine hydrochloride 2 mg, p-aminobenzoic acid 0.2 mg, biotin 10 μg, glucose and sodium acetate 10.0 g each, dipotassium phosphate 1.0 g, magnesium sulfate 100 mg, manganese sulfate 30 mg, ferrous sulfate and sodium chloride 10 mg each, Tween 80 0.5 ml, and Bacto agar 18 g.

For *Lb. casei* the complete medium consisted of this common part supplemented with 500 mg glutamic acid per litre medium. The pH of the medium was adjusted to 5.5. The *Lb. casei* medium is not perfect in all respects. There is a faint growth in the whole plate and some test solutions give diffuse rings. However, all the standard substances give dense and quite sharp rings, especially in concentrations greater than about 0.1 μg per ml. The development of an improved assay medium for *Lb. casei* is in progress in this laboratory.

For *L. citrovorum* the complete medium consisted of the common part supplemented with 10 μg folic acid per litre medium. The pH of the medium was adjusted to 6.8.

During the later course of this investigation it was found that ascorbic acid greatly improved the sharpness of the rings in the *L. citrovorum* plates. Ascorbic acid also eliminated the double ring effect that often occurred with test solutions and sometimes even with the standard substance, folinic acid. Because of the instability of ascorbic acid, it was added (as a solution) to the medium immediately before the plates were made. Ascorbic acid was used in an amount of 1 mg per ml medium. Addition of the same amount of ascorbic acid to *S. faecalis* plates caused no improvement and in *Lb. casei* plates there was a slight deterioration.

Paper chromatography.—Paper chromatography by the descending technique was carried out on 19 × 57 cm sheets of Whatman No. 1 filter paper. On these large sheets two standard solutions and 6–7 samples were run simultaneously. Three different solvent systems were employed: (I) A mixture of water-saturated sec. butanol containing 3% acetic acid; (II) a 5% aqueous solution of disodium phosphate (12 aq) in *iso*-amylalcohol according to CARTER [21] (only the aqueous phase was used in some experiments); (III) a 0.2 C sodium acetate buffer of pH = 4.0 in *iso*-amylalcohol (both phases were always used).

The chromatograms were run at 21° C for 7–24 h depending on the solvent system used. When the solvent front had advanced to about 40 cm from the starting line the chromatograms were dried in a stream of warm air (40° C) and cut to fit on the agar plates.

The R_F-values of the different growth factors often vary with the amount of sample put on the chromatograms, as previously shown by WINSTEN and EIGEN [12]. Usually 1–3 small drops from a glass capillary per spot was used. Larger amounts of a sample gave lower R_F-values in most instances. Sometimes two factors with closely related R_F-values coincided to the site of the factor with the lowest R_F-value when chromatographed in larger amounts. This was specially the case when solvent system I was used. The R_F-values given in this paper are weighted mean values. The R_F-values of the standard substances in the three solvent systems are shown in Table I.

In some experiments it was desired to study only one factor present in an extract containing several factors. The extract was then chromatographed as a band on a broad paper (24 cm) and the growth factor in question—the position of which was determined microbiologically—was eluted with distilled water.

Table I. R_F-values of the standard substances in solvent systems I, II and III.

Standard substances	Solvent system		
	I	II	III
Pteroic acid	0.10	0.05	0
Pteroylglutamic acid	0.25	0.30	0.25
Pteroyltriglutamic acid	0.20	0.58	0.58
N_{10}-formyl pteroic acid	0.62	0.62	0.62
N_{10}-formyl pteroylglutamic acid	0.45	0.80	0.80
N_5-formyl tetrahydropteroylglutamic acid	0.53	0.73	0.73
N_5-formyl tetrahydropteroyldiglutamic acid	0.35	0.83	0.83
N_5-formyl tetrahydropteroyltriglutamic acid	0.22	0.90	0.90

Paper ionophoresis.—Ionophoresis on paper was carried out as previously described [22], using Whatman No. 1 filter paper. Both the free hanging paper technique and paper placed between glass plates smeared with silicone grease were tried. The latter method gave the best results. The voltage applied was 700 volts. Phosphate buffers (0.02 C) covering a pH-range from 2.0 to 11.5 were employed. The ionophoresis experiments were run at room temperature for 2–7 h. On the whole, paper ionophoresis did not prove to be superior to paper chromatography for the separation of the *S. faecalis* factors. These exist in large numbers and all of them tend to migrate towards the anode due to their acidic properties. However, ionophoresis is valuable as a complement to chromatography for the identification of factors for this and other microorganisms. In some experiments, paper chromatography and paper ionophoresis were combined.

Bioautography.—The same test organisms and media as described above were used for the bioautographic studies. The separation of the various growth factors was accomplished, as has already been mentioned, by both paper chromatography and paper ionophoresis. After drying, the paper chromatograms and paper ionophograms were spread out on the surface of the agar plates and left there during incubation overnight at 37° C.

The incorporation of ascorbic acid into the *L. citrovorum* medium greatly improved the bioautography. In several instances it caused very good development of growth factors for *L. citrovorum* that could otherwise hardly be detected. It should be pointed out that no new growth factors appeared on incorporation of ascorbic acid in the medium. Bioautography with *S. faecalis* was also somewhat improved by addition of ascorbic acid to the medium. In contrast to this, addition of cysteine (1 mg per ml medium) or cysteine plus ascorbic acid resulted in less dense and less defined spots of growth.

Preparation of chicken pancreas and hog kidney enzymes.—Frozen chicken pancreas[1] and frozen hog kidneys obtained from freshly slaughtered animals were kept frozen until required. For preparation of enzyme stock solutions, 1 part of tissue was homogenized in a Waring blendor for 5–10 minutes in 4 parts of distilled water. After autolysis for 6–7 h at room temperature under toluene, the autolysates were frozen (−20° C). Next day the autolysates were thawed, centrifuged and filtered and the resulting enzyme solutions were kept in bottles as stock solutions at −20° C until needed. In most experiments the two enzyme preparations were used in a dilution of 1:20; thus about 10 mg tissue per ml incubation mixture was used. The blanks of both enzyme preparations in this dilution were insignificant in comparison to the amounts of growth factors enzymatically liberated. For all three test organisms the growth effect of the blanks of the diluted enzyme homogenates was of the order of 0.02 μg standard substance per ml.

In order to find out if it is desirable to protect the conjugase activity during homogenization in the Waring blendor, hog kidney was homogenized in 0.02 C cysteine hydrochloride instead of distilled water, employing the same procedures as above. However, this did not result in any higher enzyme activity, but had the disadvantage that the enzyme blank became much higher (a ten-fold increase).

Enzyme substrate.—Bacto yeast extract (Difco) was used as substrate for studying the enzymatic reactions caused by chicken pancreas and hog kidney conjugases. The amounts of free growth factors present in the yeast extract before enzymatic treatment, expressed as μg per g yeast extract, as tested for all three test organisms were: for *S. faecalis* 0.5 μg folic acid, for *Lb. casei* 35 μg folic acid, and for *L. citrovorum* 1 μg folinic acid. The high value for *Lb. casei*, owing to the greater ability of this organism to utilize higher conjugates of folic and folinic acid, should be noted.

Standard substances.—In the cup plate assays the sodium salt of folic acid (pteroylglutamic acid, Folvite Solution, Lederle) was used as standard substance for *S. faecalis* and *Lb. casei*. Synthetic folinic acid (N_5-formyl,5,6,7,8-tetrahydroformylfolic acid, Leucovorin, Lederle) was used as standard substance for *L. citrovorum*. A concentration range of 0.01 to 1.0 μg per ml was employed for both standard substances.

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[1] Obtained from full grown white Leghorn chickens.

For bioautography, 1 drop each of the following solutions of standard substances were employed:

for *S. faecalis* pteroic acid (7–9113 Lederle; 10 μg/ml), folic acid (Folvite, Lederle; 0.3 μg/ml), pteroyltriglutamic acid (Teropterin 7–9111, Lederle; 10 μg/ml), N_{10}-formyl folic acid (Ro 1-5681, Hoffman-La Roche; 0.3 μg/ml), N_{10}-formyl pteroic acid (Rhizopterin 7–9112, Lederle; 0.1 μg/ml) and folinic acid (Leucovorin, Lederle; 0.3 μg/ml),

for *Lb. casei* folic acid (0.3 μg/ml), pteroyltriglutamic acid (3 μg/ml), N_{10}-formyl folic acid (0.3 μg/ml) and folinic acid (0.3 μg/ml), and

for *L. citrovorum* folinic acid (0.3 μg/ml).

Synthetic substances [23] believed to be the di- and triglutamates of N_5-formyl, 5,6,7,8-tetrahydropteroic acid were also used as chromatographic standards for the three test organisms. The pteroyltriglutamic acid preparation used contained significant amounts of folic acid, as revealed by bioautography with *S. faecalis* and *Lb. casei.*

Synthetic factors.—Some new growth factors for *S. faecalis*, *Lb. casei* and *L. citrovorum*, prepared by formylation and catalytic hydrogenation of pteroic acid (7–9113 Lederle) and pteroyltriglutamic acid (Teropterin 7-9111, Lederle), according to the procedures previously described [23] were also studied.

Incubation procedure.—The enzyme incubations were made in small test tubes in a 37° C water thermostat. A buffer concentration of 0.1 C was used in all incubation mixtures to assure constant pH. Acetate, phosphate and ammonia-ammonium chloride buffers were used. The toluene layer covering the reaction mixture in experiments when incubating overnight, was omitted in the case of some short time incubations (1 h). All enzyme reactions were stopped by heating in a boiling water bath for 5 minutes.

For examining the effect of pH on the ability of the chicken pancreas and hog kidney enzymes to liberate growth factors from yeast extract, incubation mixtures containing 20 mg yeast extract and 10 mg enzyme tissue per ml in 0.1 C buffer of the appropriate pH were made. The pH of the incubation mixtures were controlled before and after incubation. No change of pH occurred in any experiment. The total volume of the incubation mixtures was either 2.1, 4 or 8 ml. Acetate buffers were used for pH 3, 3.5, 4, 4.5, 5, 5.5, 6, 7 and 8, phosphate buffers for pH 5, 6, 7, 8 and 9, and ammonia-ammonium chloride buffers for pH 8, 9, 10 and 11.

Results

Effect of pH on the activity of chicken pancreas conjugase.—Four series of experiments with the incubation mixtures described above were carried out. In each series the pH was varied from 3 to 11.

The two first series in which all three types of buffers were used were carried out simultaneously. In series II—but not in series I—cysteine hydrochloride was added to a concentration of 0.01 C. The incubation mixtures, 8 ml, were covered with a layer of toluene. Portions (2 ml) were withdrawn after incubation for 1 h and 22 h respectively. The pH was adjusted to 6.8–7.2 with an equal volume of 0.1 C phosphate buffer of appropriate composition, before stopping the enzyme action by boiling.

The two last series (series III and IV) were also carried out simultaneously. Only the acetate and ammonia-ammonium chloride buffers were used. In series IV—but not in series III—ascorbic acid was added to a concentration of 0.01 C. The mixtures, 2.1 ml, were incubated for 1 h at 37° C. No toluene was employed and no adjustment of the pH of the incubation mixtures was made after the incubation before the enzyme action was stopped by boiling.

The enzymatically released growth factors were determined with the cup plate and the bioautographic methods, using all three test organisms. In order to compare the curves for the effect of pH on the activity of the enzyme, the highest activity obtained for each test organism (*S. faecalis*, *Lb. casei* and *L. citrovorum*) within a series was given the value 100. The other values of the activities for each test organism were expressed in percent thereof. The highest enzyme activity (100) expressed as μg liberated growth factor per g yeast extract were for the the three test organisms as follows: for *S. faecalis* 50 μg folic acid, for *Lb. casei* 50 μg folic acid, and for *L. citrovorum* 200 μg folinic acid. All four series (I–IV) gave the same values of maximal activity. No difference could be observed between samples incubated for 1 h and 22 h (series I and II). The enzymatic action is thus completed within 1 h under the prevailing experimental conditions..

As mentioned before, yeast extract gives a very high blank for *Lb. casei*, an organism known to respond to higher conjugates of folic acid. The growth promoting effect of yeast extract towards *Lb. casei* is consequently only moderatly increased (about 40%) on enzymatic treatment. Furthermore, the cup plate method using *Lb. casei* is not perfect in all respects and the results with this test organism are therefore somewhat uncertain. Nevertheless the activity-pH curve obtained when *Lb. casei* served as test organism appears to have a similar trend as the curves obtained with *S. faecalis* and *L. citrovorum*. Bioautography with *Lb. casei* gave good results and revealed, using solvent system I, that the high blank of yeast extract for *Lb. casei* is due to one large spot, hardly moving from the starting point.

The two series in which no addition of cysteine or ascorbic acid was made (series I and III) and the series with addition of 0.01 C ascorbic acid (series IV) gave essentially the same pH-curves for the enzyme activity, as determined with the cup plate method using *S. faecalis* and *L. citrovorum* (Fig. 1). Chicken pancreas homogenate exhibits a very broad pH-range for enzyme activity, optimum release being obtained at pH-values varying from about 4.5 to 8.5. The results with *Lb. casei* indicated that the activity-pH curve as determined with this organism resembled the curves obtained with *S. faecalis* and *L. citrovorum*, showing a maximum at about 5.0. In the presence of 0.01 C cysteine hydrochloride (series II) the pH-curve, as determined with all three test organisms, was somewhat narrower, the pH-optimum now being 5.0 to 7.5. A small liberation of growth factors occurred at pH 4.0 but almost none at pH 9.0.

Bioautography with solvent system I revealed that the growth promoting effect for *S. faecalis*, *Lb. casei* and *L. citrovorum* of the samples in series I and II was caused by the same three growth factors, no matter whether the samples had been incubated for 1 h or for 22 h. Two of these factors occurred only in small amounts and had the R_F-values 0.22 and 0.53 respectively. The R_F-value of the third factor, which was responsible for most of the effect, was 0.35. The factor with R_F 0.53 (folinic acid) was observed occasionally and only in samples that had been incubated at pH-values lower than 5.5. The R_F-values 0.22 and 0.35 of the other two factors are the same as those of the synthetically prepared tri- and diglutamates of N_5-formyl tetrahydropteroic acid (see Table I) which we found to be active for all three test organisms. The identity of the synthetically prepared compounds with those obtained from enzymatically treated yeast extract was confirmed further by chromatographic and ionophoretic studies. The triglutamate (R_F 0.22) was

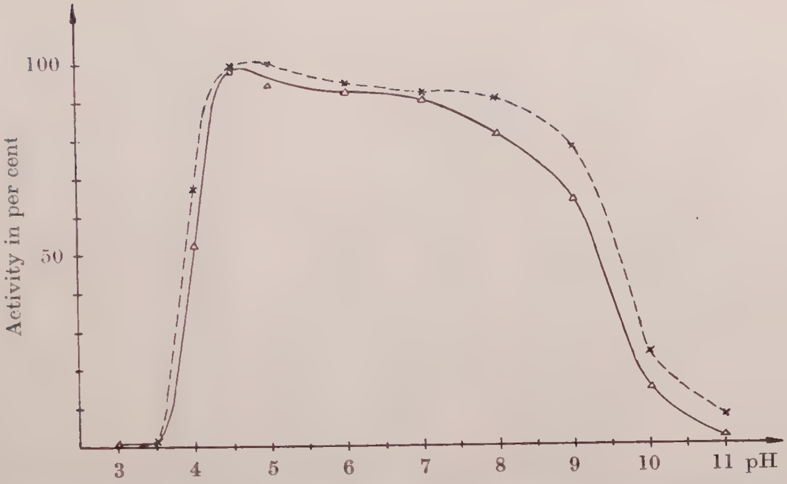

Fig. 1. Effect of pH on the enzymatic release of growth factors for *S. faecalis* (—) and *L. citrovorum* (---) from yeast extract by means of chicken pancreas homogenate. Incubation at 37° C for 1 h. When *Lb. casei* was used as test organism the activity-pH curve showed a similar trend.

present only in samples that had been incubated at pH-values lower than 5.5 or higher than 9.0.

Bioautographic studies, using solvent system I, of the samples in the series III and IV showed that the total growth promoting effect of these samples was due to four factors all of which seemed to be active for all of the three test organisms employed. The main factor appeared to be the same as in the samples of the series I and II, i.e. the diglutamate of N_5-formyl tetrahydropteroic acid (R_F 0.35). The second factor had R_F 0.22 and was found to be the triglutamate of N_5-formyl tetrahydropteroic acid. In agreement with the findings on the samples of series I and II it was observed that this acid was present only at pH-values near the boundaries of or outside the optimum pH-range of chicken pancreas. The third factor had R_F 0.08. A factor with this R_F has been obtained also by chemical conversion of folinic acid as will be described later. We believe that this factor is identical with the "Factor I" present in marine algae [20, 23], and will therefore be referred to as Factor I in the present paper. Factor I is present only in the samples which have been incubated at pH-values lower than 5.5. It disappeared when the pH of these samples was adjusted to about 7 (with sodium hydroxide or phosphate buffer). This explains the absence of Factor I in the samples of the series I and II in which the pH was taken to about 7 after the incubation. The fourth factor present in the samples of series III and IV was found to be folinic acid. Folinic acid occurred only in minute amounts and appeared, as did Factor I, only in samples which had been incubated at pH-values lower than about 5.5.

It should be mentioned that the nature of the buffer used in the incubation mixture did not seem to influence the types of the factors formed.

The results of this investigation indicate that the end product of the action of chicken pancreas conjugase on higher conjugates of folinic acid is mainly the diglutamate of N_5-formyl tetrahydropteroic acid.

Effect of pH on the activity of hog kidney conjugase.—Four series (V–VIII) of

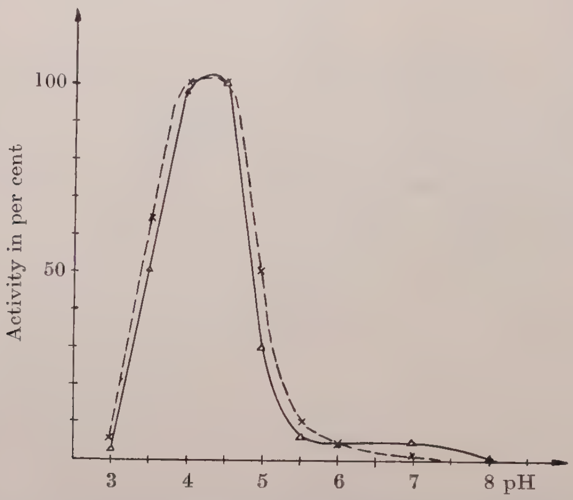

Fig. 2. Effect of pH on the enzymatic release of growth factors for *S. faecalis* (—) and *L. citrovorum* (- - -) from yeast extract by means of hog kidney homogenate. Incubation at 37° C for 1 h.

experiments with the incubation series described above, in which the pH was varied from 3 to 11, were carried out simultaneously using the acetate and ammonia-ammonium chloride buffer series. The mixtures, 2.1 ml, were incubated for 1 h at 37° C. No toluene was used and no adjustment of the pH of the incubation mixtures was made after the incubation before the enzyme action was stopped by boiling.

In series V, VI and VII a homogenate of hog kidney in water, in series VIII a homogenate of hog kidney in 0.02 C cysteine hydrochloride was used as enzyme preparation. The incubation mixtures in series V and VIII contained only yeast extract and hog kidney homogenate. In series VI also cysteine hydrochloride and in series VII ascorbic acid was added to a concentration of 0.01 C. The highest release of growth factor, given the arbitrary value 100, corresponded per g yeast extract to 50 μg folic acid for *S. faecalis* and to 200 μg folinic acid for *L. citrovorum*. *Lb. casei* was not used in these experiments.

All four series (V–VIII) gave approximately the same values for maximal enzyme activity and essentially the same curves for the effect of pH on the enzyme, as determined with *S. faecalis* and *L. citrovorum* (Fig. 2). The only difference observed was that a comparatively high enzyme blank was obtained when the hog kidney was homogenized in the presence of cysteine hydrochloride (series VIII). Hog kidney conjugase appears to be active only at pH-values between 3 and 7. A sharp maximum in the curve appeared at pH 4 to 4.5. Bioautography of samples incubated for 1 h revealed the presence of the following factors: Factor I, the di- and triglutamate of N_5-formyl tetrahydropteroic acid, folinic acid and folic acid. The first four factors are the same as those observed in the incubation mixtures with chicken pancreas. However, in the case of hog kidney the spot due to folinic acid was the most intensive. At lower pH, Factor I contributed more to the total effect than at higher pH. The same five factors appeared even it the incubation period was prolonged to 20 h. Hog kidney homogenate has been observed to degrade synthetically prepared di- and triglutamates of N_5-formyl tetrahydropteroic acid to folinic acid

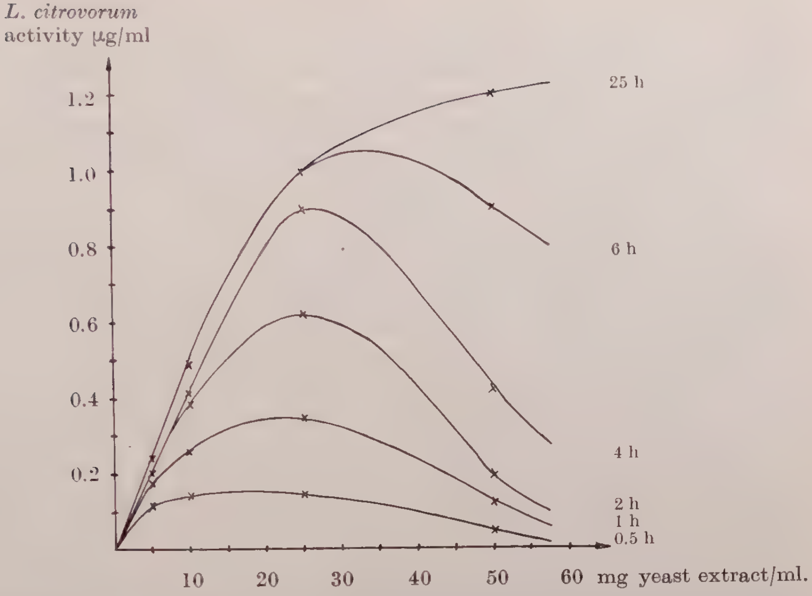

Fig. 3. Effect of the time of incubation and the concentration of the yeast extract on the enzymatic release of growth factors for *L. citrovorum* from yeast extract by hog kidney homogenate at pH 4.5 (acetate buffer).

(see below). The inability of hog kidney homogenate to completely break down higher conjugates of yeast extract to folinic acid is probably due to the presence of conjugase inhibitors in this material (see Fig. 3).

The effect of incubation time and of substrate concentration on the amount of enzymatically released growth factors by hog kidney homogenate was also investigated. *L. citrovorum* served as test organism and the pH of the incubation mixture was 4.5. As shown in Fig. 3, maximum release of growth factors for *L. citrovorum* occurred at a ratio of hog kidney tissue to yeast extract of about 0.60 when the time of incubation was shorter than 4 h. The experiments demonstrate the presence of substances inhibiting the action of hog kidney homogenate: an increase in the amount of yeast extract acted upon by a given amount of hog kidney enzyme caused a decrease in the release of growth factors for *L. citrovorum*.

Pteroyltriglutamic acid, formylated and catalytically hydrogenated.—Pteroyltriglutamic acid, active only for *S. faecalis* and *Lb. casei*, becomes highly active also for *L. citrovorum* after formylation and catalytic hydrogenation [13, 23]. Bioautographic studies using solvent system I revealed that the growth promoting effect of formylated and reduced pteroyltriglutamic acid for *L. citrovorum* was due to three different growth factors with the R_F-values 0.22, 0.35 and 0.53. These three growth factors were also active for *S. faecalis* and *Lb. casei*. The growth factor with R_F 0.22 was responsible for most of the activity as measured with *Lb. casei* and *L. citrovorum*. Tested with *S. faecalis*, the spots of the three factors had about the same intensity. The growth factor with R_F 0.53 is folinic acid. The presence of this acid in the reaction mixture is at least partly due to the presence of folic acid in the starting material. The growth factors having the R_F-values 0.22 and 0.35

appear to be the triglutamic acid and diglutamic acid derivatives of N_5-formyl, 5,6,7,8-tetrahydropteroic acid respectively.

When chromatographing formylated and catalytically hydrogenated pteroyltriglutamic acid in high concentration, three more growth factors for *S. faecalis* were detected. The first factor had the same R_F-value as N_{10}-formyl pteroic acid (R_F 0.62) and the second, which is a new growth factor for *S. faecalis*, had R_F 0.70. The third growth factor was active for *S. faecalis*, *Lb. casei* and *L. citrovorum* and had R_F 0.08, i.e. the same as Factor I.

Some experiments were carried out with the two growth factors having the R_F-values 0.22 and 0.35, presumed to be the tri- and diglutamates of N_5-formyl tetrahydropteroic acid which had been separated in small quantities by preparative chromatography. Digestions of the di- and triglutamates with chicken pancreas and hog kidney homogenates clearly demonstrated the different action of the enzyme systems of these two organs. Digestion of the triglutamate with chicken pancreas homogenate gave rise mainly to the diglutamate. In agreement with this finding it was observed that the enzyme system of chicken pancreas had no significant effect on the diglutamate. On the other hand, the main product formed on digestion of both the di- and the triglutamates with hog kidney homogenate was folinic acid.

Pteroic acid, formylated and catalytically hydrogenated.—Pteroic acid, formylated and catalytically hydrogenated yields quite a good activity for *S. faecalis* and some activity for *Lb. casei* [13, 23]. Only slight activity for *L. citrovorum* was found, in agreement with the findings of SHIVE *et al.* [13]. Bioautography with *S. faecalis* using solvent system I revealed that most of the growth promoting effect was caused by the new growth factor mentioned above, having the R_F-value 0.70. The rest of the *S. faecalis* activity was caused (in order of decreasing intensity) by N_{10}-formyl pteroic acid, folinic acid, pteroic acid and two factors having lower R_F-values than that of pteroic acid. The formation of folinic acid was probably due to the presence of small quantities of folic acid in the pteroic acid used. The new growth factor with R_F 0.70 was found to be inactive for *Lb. casei*. Bioautography (solvent system I) revealed that the growth of *Lb. casei* was caused by folinic acid. Small quantities of the new growth factor (R_F 0.70), active only for *S. faecalis*, were separated by preparative chromatography. Paper ionophoresis of this growth factor in phosphate buffer with pH 8.5 showed that it migrated considerably faster than pteroic acid but slower than N_{10}-formyl pteroic acid. Paper chromatography using solvent systems II and III gave R_F-values for the new factor of 0.65 and 0.50 respectively.

Chemical conversions of folinic acid.—In order to study the alkali and acid stability of folinic acid (Leucovorin) 1 ml portions (30 μg/ml) were treated with 4 ml of respectively:

a) 0.1 C NaOH resp. HCl, at 37° C for 2 h.
b) 0.1 C NaOH resp. HCl, at 120° C for 15 min.
c) 0.1 C NaOH resp. HCl, at 120° C for 1 h.
d) 1.0 C NaOH resp. HCl, at 120° C for 15 min.

The samples were cooled in ice-water, neutralized immediately and diluted for assay, using *S. faecalis* and *L. citrovorum*. As seen from table II treatment with 0.1 C and 1.0 C alkali caused a comparatively slight decrease in the growth promoting effect of Leucovorin for *S. faecalis*. Nearly all the effect for *L. citrovorum* was destroyed by 1.0 C alkali. Acid treatment destroyed the effect of Leucovorin for *L. citrovorum* more rapidly than for *S. faecalis*. Bioautography, employing solvent

Table II. Remaining growth promoting activity of folinic acid (Leucovorin) for *S. faecalis* and *L. citrovorum* after treatment with alkali and acid (in per cent of untreated Leucovorin). Bioautography with solvent system I. Bold-face type for an R_F-value indicates that the corresponding growth factor is active for both *S. faecalis* and *L. citrovorum*. Ordinary type indicates activity only for *S. faecalis*. When KOH was used instead of NaOH, Factor I, folic acid, N_{10}-formyl folic acid and Leucovorin were present in all four alkaline reaction mixtures.

Treatment with	Activity for		R_F values for growth factors present
	S. faecalis	*L. citrovorum*	
0.1 C NaOH, 37° C, 2 h	85	70	**0.08** (Factor I) 0.45 (N_{10}-formyl folic acid) **0.53** (Leucovorin)
0.1 C NaOH, 120° C, 15 min.	100	80	0.25 (folic acid) **0.53** (Leucovorin)
0.1 C NaOH, 120° C, 1 h	65	40	0.25 (folic acid) **0.53** (Leucovorin)
1.0 C NaOH, 120° C, 15 min.	75	5	**0.08** (Factor I) 0.45 (N_{10}-formyl folic acid)
0.1 C HCl, 37° C, 2 h	60	2	0.45 (N_{10}-formyl folic acid)
0.1 C HCl, 120° C, 15 min.	20	1	—
0.1 C HCl, 120° C, 1 h	1	0	—
1.0 C HCl, 120° C, 15 min.	5	1	—

system I, revealed that alkali treatment of Leucovorin gave rise to three other growth factors for *S. faecalis*, having the R_F-values 0.08, 0.25 (folic acid) and 0.45 (N_{10}-formyl folic acid). The factor with R_F 0.08 is active also for *L. citrovorum* and is probably identical with Factor I. Bioautography revealed that N_{10}-formyl folic was formed intermediately also during the acid breakdown of Leucovorin.

Pteroic acid, folic acid, N_{10}-formyl folic acid and N_{10}-formyl pteroic acid were treated in the same way as Leucovorin. It was then observed that pteroic acid was very stable to alkali and acid treatment. Folic acid was stable to alkali but was partly destroyed by acids. N_{10}-formyl pteroic acid and N_{10}-formyl folic acid were rapidly deformylated by alkali or acids to pteroic acid and folic acid respectively.

The breakdown of Leucovorin in alkali was also studied by heating 20 ml Leucovorin (30 µg/ml) with 80 ml 0.1 C NaOH and 0.1 C KOH respectively under reflux in a boiling water bath for 24 h. Samples (1 ml) were withdrawn after 0.5, 1, 2, 4, 8, and 24 h, neutralised and diluted for assay. The course of the breakdown of Leucovorin in 0.1 C KOH, as followed with *S. faecalis* and *L. citrovorum*, is shown in Fig. 4. As seen from the figure, the growth promoting activity for *L. citrovorum* was destroyed more rapidly than that for *S. faecalis*. The rather unexpected observation was made that the activity of Leucovorin for both *S. faecalis* and *L. citrovorum* appeared to be higher after 1 than after 0.5 h boiling in 0.1 C KOH. All samples contained the same four growth factors for *S. faecalis*—two of which were active also for *L. citrovorum*—as observed in the other experiments with alkali treatment described. Similar but less regular results were obtained when NaOH was used instead of KOH.

Leucovorin was also boiled in solutions containing reducing agents, e.g. ascorbic acid, cysteine hydrochloride, glutathione and thioglycolic acid. The interesting observation was made that all these compounds caused conversion of Leucovorin

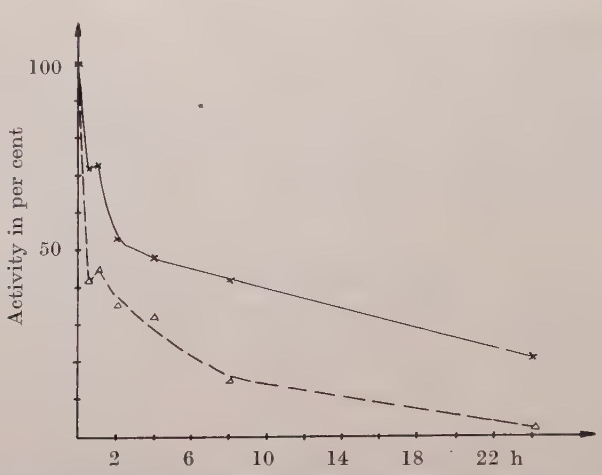

Fig. 4. Decrease in growth promoting effect of Leucovorin for *S. faecalis* (—) and *L. citrovorum* (---) when boiled in 0.1 C KOH.

to Factor I (R_F 0.08), N_{10}-formyl folic acid and folic acid. Factor I was obtained in good yields and was formed also when Leucovorin was boiled at pH 4.5 in the presence of serine. Formate did not give Factor I.

Discussion

In the present investigation chicken pancreas conjugase has been shown to exhibit a wide range of activity from pH 3.5 to 10.5 for the enzymatic liberation of growth factors for *S. faecalis*, *Lb. casei* and *L. citrovorum* from crude yeast extract. The enzyme has a broad pH-optimum of 4.5 to 8.5. Slightly more activity for all three test organisms is liberated at about pH 4.5–5 than at any other pH-value. This is contrary to earlier reports in the literature. BIRD *et al.* [24], for instance, obtained a pH-optimum of 7.0 to 7.5, with very little enzyme activity occurring at pH 4.5, for the enzymatic liberation of growth factors active for *S. faecalis* from crystalline pteroylheptaglutamic acid and from a purified preparation of this compound from yeast extract. A crude homogenate of chicken pancreas in phosphate buffer served as enzyme substrate. LASKOWSKI *et al.* [25] also obtained a pH-optimum of 7. They used a crude water extract of chicken pancreas as enzyme source and crude yeast extract as substrate. With purified enzyme preparations the optimal activity of the enzyme occurred at pH 8. Using a purified chicken pancreas conjugase preparation as enzyme source and crystalline pteroylheptaglutamic acid or a concentrate of this factor from yeast as substrate, MIMS *et al.* [26] obtained the pH-optimum of 7.8 for the enzymatic release of growth factors for *S. faecalis*. Very little activity occurred at pH 4.5. On the other hand, BURKHOLDER *et al.* [27] obtained a pH-optimum of 4.5 to 5.0 for the enzymatic release of growth factors for *Lb. casei* using a phosphate buffer homogenate of chicken pancreas as enzyme source. Three types of yeast extract powder served as substrates. In all the investigations mentioned [24–27] the assays were carried out by means of the tube method, using *S. faecalis* or *Lb. casei*. FORD [30] has recently shown that different vitamin B_{12}-factors give different microbiological response in the tube assay and in the agar cup plate method. It is probable that a similar phenomenon is at least partly the cause for the differences between our results and those of other authors.

When chicken pancreas homogenates or purified preparations thereof have been used for the enzymatic release of growth factors for *S. faecalis*, *Lb. casei* and *L. citrovorum* from natural sources, a pH of 7 to 7.5 has been used by most investigators [8, 9, 16, 17, 20, 28, 29]. In this laboratory a pH slightly less than 4 has been used for the liberation of growth factors for *S. faecalis* and *L.citrovorum* from marine algae [23]. At this low pH equal amounts of growth factors for these test organisms were liberated as at pH 7.5 [20]. As a consequence, when using chicken pancreas conjugase for the liberation of growth factors for *S. faecalis*, *Lb. casei* and *L. citrovorum* from natural sources, incubations at several different pH-values must be tried in each case, in order to secure maximal release.

Our results with hog kidney homogenate which revealed a narrow pH-optimum from 4.0 to 4.5 are in accordance with earlier findings in the literature [24, 31].

The very broad pH-optimum of chicken pancreas conjugase may indicate that several different natural substances are acted upon by a single unspecific enzyme or by a mixture of several different enzymes. Studies with several synthetic substances reported by DABROWSKA *et al.* [32] indicated that chicken pancreas might be one single, unspecifically working enzyme. However, other reports in the literature have suggested that the action of enzyme preparations obtained from various sources not only involves liberation of the various growth factors but also interconversions of these factors, e.g. formylation, deformylation, transformylation and hydrogenation. The findings of NICHOL and WELCH [33–35] using rat liver, RAUEN *et al.* [36, 37] using liver homogenates of various animals, DIETRICH *et al.* [38], using chick liver, ERICSON *et al.* [20] using chicken pancreas homogenates, BOND [39] and BROQUIST *et al.* [40] using cells of *Lb. casei* and of *S. faecalis*, indicate the existence of a number of different enzymes acting on folic acid and related factors. Preliminary experiments in this laboratory indicate that also hog kidney homogenates may carry out not only liberation but also conversions of various factors active for *S. faecalis*, *Lb. casei* and *L. citrovorum*.

The different effect of cysteine hydrochloride upon the activity-pH curves for chicken pancreas and hog kidney homogenates indicate that the enzymes of these two organs differ from each other. In contrast to the findings of other authors [14, 26, 41], no activating effect of cysteine hydrochloride on hog kidney was found in our experiments. This is probably due to the low concentration of cysteine hydrochloride used.

The enzyme(s) acting in hog kidney, giving folinic acid as the main end-product, showed optimal activity at pH 4.0 to 4.5. Although the enzyme of chicken pancreas is very active in this pH-range, its main end-product is not folinic acid but the diglutamate of N_5-formyl tetrahydropteroic acid. This demonstrates that different enzymes (*inter alia*, conjugases) are present in hog kidney and chicken pancreas. Furthermore, the experiments with the synthetically prepared di- and triglutamates of N_5-formyl tetrahydropteroic acid as substrates for studying the action of hog kidney and chicken pancreas again showed the different nature of these enzymes.

Of the results obtained by acid or alkali treatment of various factors related to folinic acid or folic acid, the findings with folinic acid (Leucovorin) deserve special attention. ANDREWS and SCHWEIGERT [9] have also studied the chemical stability of Leucovorin but their results differ from ours. According to our findings hydrolysis of Leucovorin under mildly acidic conditions results in the formation of N_{10}-formyl folic acid. This is in agreement with the findings of May *et al.* [42] and of Cosulich *et al.* [5]. Alkali treatment gives rise to a factor, other than folinic acid, which is active for *L. citrovorum* and *S. faecalis* as well as for *Lb. casei*, i.e. Factor I, and also to factors active only towards the two last-mentioned organisms, i.e. N_{10}-formyl folic acid and folic acid. Factor I can also be obtained by treatment of Leucovorin with various reducing agents or—rather unexpectedly—with serine but not with formate. The formation of Factor I on chemical treatment of Leucovorin is of interest as a compound, with the same ionophoretic and chromatographic properties, and active towards the same three microorganisms, as found after enzymatic digestion of extracts of algae [20] and of yeast. The Factor I obtained from Leucovorin, however, differs in stability from the factor obtained on enzymatic treatment, the former but not the latter being stable at neutral or alkaline pH-values. The factor formed by chemical treatment of Leucovorin may naturally be a mixture of different stereoisomers, whereas the factor resulting from enzymatical digestion is likely to be one particular stereoisomer. Factor I has been observed to occur in aqueous extracts of marine algae [23] and may therefore occur as such in Nature. The fact that Factor I is active towards *Lb. casei*, further that no factor for *L. citrovorum* appears to be formed on formylation and hydrogenation of pure pteroic acid, and finally that Factor I can be obtained by relatively mild treatment of Leucovorin makes it very unlikely that Factor I should be formylated and hydrogenated pteroic acid or a close derivative thereof. It seems more probable that Factor I is a derivative of folinic acid. The acidic properties of Factor I appear to be weaker than those of folinic acid.

The existence of a large number of factors for *S. faecalis*, some of which were presumed to be active also towards *L. citrovorum*, was reported in a previous paper from this laboratory [20]. The results of the present investigation and of separate studies on marine algae [23] have verified these earlier findings. One further factor active towards *S. faecalis* has now been found. This factor which is a derivative of pteroic acid, has not as yet been found in any natural material but has been obtained only by formylation and reduction of pteroic acid. This treatment of pteroic acid would be expected to give N_5-formyl tetrahydropteroic acid which may be the structure of this factor. This would be in accordance with its behaviour in solvent systems II and III (R_F 0.65 and 0.50 respectively) in which the R_F-values decrease with decreasing number of glutamic acid residues in the compounds (see Table I). If the proposed formula of the factor is correct, it is of interest to notice that a compound with this structure is inactive for *L. citrovorum*. On the whole, it appears as if *L. citrovorum* responds to higher conjugates of folinic acid in a way similar to the response of *Lb. casei* to higher conjugates of folic acid (and folinic acid).

The low specificity of *S. faecalis* makes it unlikely that the values for folic acid activity of various materials given in the literature should reflect the true folic acid content of the substances tested. Moreover, very little folic acid (pteroylglutamic acid) seems to be present in most natural materials [7, 19, 20, 23]. The *S. faecalis*-activity of yeast is, as has been shown in this paper, due mainly to derivatives of folinic acid. The factors occurring in marine algae [20, 23] or in lichens [19] appear also to be derivatives of folinic acid and formylated forms of pteroic acid and folic acid.

SUMMARY

The effects of the enzyme systems of chicken pancreas and hog kidney homogenates on yeast extract and on synthetically prepared conjugates of folinic acid have been investigated. Chemical degradation studies on folinic acid and on other growth factors related to this compound and to folic acid have been undertaken. *Streptococcus faecalis*, *Lactobacillus casei* and *Leuconostoc citrovorum* have served as test organisms in the agar cup plate method. Bioautographic studies using chromatography and ionophoresis on paper separately and in combination have been carried out.

It has been shown that the enzyme system of chicken pancreas homogenate exhibits a very broad pH-optimum, maximum release being obtained from pH 4.5 to 8.5. This is in contrast to the enzyme activity of hog kidney homogenate the pH-optimum of which was found to lie between pH 4.0 and 4.5. Ascorbic acid (0.01 C) did not seem to influence the activity of the enzymes of either chicken pancreas or hog kidney homogenate. Cysteine hydrochloride (0.01 C), on the other hand, diminished the pH-region for optimal activity of the enzyme system of chicken pancreas homogenate but appeared to be without effect on the enzymes of hog kidney homogenate.

The end-product of the digestion of higher conjugates of folinic acid with chicken pancreas homogenate is mainly the diglutamate of N_5-formyl tetrahydropteroic acid, whereas the main end-product of the digestion with hog kidney homogenate appears to be folinic acid. The triglutamate of N_5-formyl tetrahydropteroic acid seems to be intermediately formed in both cases. These di- and triglutamates have been found to be active for *S. faecalis*, *Lb. casei* and *L. citrovorum*.

Another growth factor active towards all three microorganisms is formed when yeast extract is treated with chicken pancreas homogenate at pH-values lower than 5.5 or with hog kidney homogenate at pH-values lower than 4.5. The same factor has been obtained by alkaline hydrolysis of folinic acid, by boiling of folinic acid in the presence of reducing agents, e.g. ascorbic acid, cysteine hydrochloride, glutathione or thioglycolic acid or in the presence of serine. The features of this factor indicate that it should be a derivative of folinic acid rather than of formylated and reduced pteroic acid. Formylation and reduction of pteroic acid give rise to a factor active only towards *S. faecalis* and with features different from those of the known growth factors for this microorganism. This last mentioned factor has as yet not been found to occur in natural material.

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Further studies on growth factors for *Streptococcus faecalis* and *Leuconostoc citrovorum* in marine algae

By L.-E. ERICSON

With 2 figures in the text

Marine algae have been shown in this laboratory to contain several different water-soluble growth factors related to the folic acid and the folinic acid groups of vitamins [1, 2]. The total activities of these factors of the algae investigated for the respective test organisms, *Streptococcus faecalis* ATCC 8043 and *Leuconostoc citrovorum* ATCC 8081, were found to be comparatively low. Only six different algae have as yet been studied in this respect. It appeared desirable to analyse a larger number of algae in order to get a better idea of the value of seaweed as a source of folic acid and folinic acid. Seventeen different marine algae representative of the four classes *Rhodophyceae*, *Phaeophyceae*, *Chlorophyceae* and *Heterokontae* as well as a sample of plankton have therefore been investigated. Some of the algae have been divided into different parts, i.e. fertile tips and vegetative thallus or into frond, stipe and holdfast, and the folic acid and folinic acid activities of these parts have been determined separately. The seasonal variations in the total folic acid and folinic acid activities in the brown algae *Alaria esculenta* and *Laminaria hyperborea* have also been studied.

It has been suggested by various authors that higher conjugates of folinic acid may exist in natural material [1–11]. Also marine algae contain substances other than folinic acid and thymidine which can replace folinic acid as growth factors for *L. citrovorum* [1, 2]. An attempt to identify some of these substances with synthetically prepared compounds has been made.

Experimental

Microbiological methods.—Folic acid activity was measured microbiologically using *Streptococcus faecalis* ATCC 8043 in an agar cup plate method. The medium consisted of the Bacto folic acid assay medium to which 1.7% Bacto agar was added. Folinic acid was determined with *Leuconostoc citrovorum* ATCC 8081 in the medium of BOLINDER *et al.* [12] using the agar cup plate method.

Bioautographic studies.—The solvent systems for the paper chromatographic separations consisted of (I) sec. butanol saturated with water and containing 3% acetic acid and (II) a mixture of *iso*-amylalcohol and a 5% solution of disodiumphosphate according to CARTER [13]. Ionophoresis on paper was carried out as described previously [14]. The chromatograms and ionograms were dried in a stream of warm air (40°C) and spread out on agar plates seeded with the microorganism in question.

Extraction procedures.—It has been shown [1] that digestion with a homogenate of chicken pancreas gives the highest values for the folic acid activity of algae. Chicken pancreas homogenate has also been found to be very effective for the liberation of compounds with folinic acid activity from algal material. This method for releasing the folic acid and folinic acid activity has therefore been used for the quantitative determinations of these vitamins (Table I). It has been observed that the ability of the enzyme(s) to liberate growth factors for *S. faecalis* and *L. citrovorum* is nearly equally great throughout the range pH 4–9 [12]. A pH slightly below 4 was used in order to prevent slimy substances from dissolving from the algal material. When determining the seasonal variation in the folic acid or folinic acid activity of *Alaria esculenta*, all samples were treated in two further ways, i.e. with a hog kidney homogenate or by autoclaving in water. The three different methods of extraction were used to obtain full evidence for the significance of certain maxima and minima in the seasonal variation. The two last extraction methods gave much lower values than that using digestion with chicken pancreas (see Fig. 1). It should be mentioned that the time required for maximum release of factors with *L. citrovorum* activity was much shorter than the time required for maximum release of factors with *S. faecalis* activity. This suggests that the folinic acid type of factors exists in algae in form(s) different from those of the folic acid factors.

Synthetic factors.—Some factors for *L. citrovorum* were prepared by formylation and catalytic hydrogenation of growth factors for *S. faecalis*. The catalyst was prepared from H_2PtCl_6 and sodium nitrate according to ADAMS *et al.* [15].

The following crystalline compounds were treated: pteroic acid (7–9113, Lederle), pteroylglutamic acid (Hoffmann-La Roche;) and pteroyltriglutamic acid (Teropterin 7–9111, Lederle). The formylation and reduction were carried out in a way similar to that described by SHIVE *et al.* [16, 17] and can be exemplified by the conversion of pteroyltriglutamic acid, active only towards *S. faecalis*, to a compound believed to be N_5-formyl, 5,6,7,8-tetrahydropteroyltriglutamic acid and active for *L. citrovorum*.

Pteroyltriglutamic acid (10.2 mg) was dissolved in 5 ml 99–100% formic acid and 0.5 ml acetic acid anhydride. The solution was kept at 40° C for 1 h in a water bath. 6.4 mg of the platinum catalyst and 27 mg ascorbic acid were then added and hydrogen was bubbled through this solution for about five hours at room temperature. 25 mg ascorbic acid was added and the solution filtered through a glass filter and evaporated to dryness in vacuo. The residue was dissolved in a 2% soda solution and autoclaved at 120° C for 1 h. The solution thus obtained was highly active for *L. citrovorum*. Bioautographic studies (solvent system I) revealed that the activity was due to three different compounds but that one of these compounds was responsible for most of the activity. This compound was not identical with N_5-formyl, 5,6,7,8-tetrahydropteroylglutamic acid (folinic acid, Leucovorin) and had the lowest R_F-value (0.22) of the three factors. One of the other factors had an R_F-value identical with folinic acid (0.53). The R_F-value of the third factor was 0.35, i.e. it was situated between the two first-mentioned factors.

When pteroylglutamic acid was treated in the same way as that just described for pteroyltriglutamic acid, a growth factor for *L. citrovorum* was obtained which had the same R_F-value as folinic acid in the two solvent systems. Its behaviour on ionophoresis on paper at pH 8.5 and at pH 11.6 was identical to that of folinic acid.

Digestion of the solution obtained on formylation and reduction of pteroyltriglutamic acid with chicken pancreas or hog kidney homogenates caused a decrease in the intensity of the strongest spot (R_F 0.22) which was followed by an increase in the two spots with R_F 0.35 and R_F 0.53 (folinic acid) respectively [12]. Hydrolysis in alkali caused destruction of the compound with R_F 0.22 and formation of folinic acid and also of glutamic acid (paper chromatographic evidence). On ionophoresis on paper the factor with R_F 0.22 was observed to move faster towards the anode than folinic acid, which is in agreement with the fact that pteroyltriglutamic acid moves faster towards the anode than pteroylglutamic acid (folic acid). This indicates the presence of a larger number of glutamic acid residues in the factor with R_F 0.22 than in folinic acid.

From these experiments it seems very likely that the factor with R_F 0.22 is N_5-formyl, 5,6,7,8-tetrahydropteroyltriglutamic acid. The factor with R_F 0.35 is likely to be N_5-formyl, 5,6,7,8-tetrahydropteroyldiglutamic acid. The presence of folinic acid in the reaction mixture is due, at least partly, to the presence of folic acid in the starting material.

Pteroic acid when submitted to formylation and reduction yielded a solution with only a slight activity. This is in accord with the findings of SHIVE *et al.* [16]. However, bioautographic studies revealed that the active principle in this solution was folinic acid. The formation of this compound must be due to the presence of small quantities of folic acid in the pteroic acid used. Therefore, either the attempt to obtain N_5-formyl, 5,6,7,8-tetrahydropteroic acid failed or this compound may not be active towards *L. citrovorum*. The latter explanation appears to be the most plausible [12]. A growth factor with R_F 0.70 was responsible for most of the *S. faecalis* activity of the formylated and reduced pteroic acid. This substance also appeared in alkali hydrolysates of the compound believed to be N_5-formyl, 5,6,7,8-tetrahydropteroyltriglutamic acid (small amounts of the factor were present before alkali treatment).

Formylation without subsequent reduction of pteroyltriglutamic acid gives rise to a number of growth factors for *S. faecalis*, among others, N_{10}-formylpteroic acid and N_{10}-formylfolic acid. The material possessed, however, no activity for *L. citrovorum*. The factors believed to be the di- and triglutamates of N_5-formyl tetrahydropteroic acid seem to be active both for *S. faecalis* and *L. citrovorum*.

Results

The total activities for *S. faecalis* and *L. citrovorum* in a number of seaweeds are given in Table I. Pteroylglutamic acid (Folvite, Lederle) was used as standard for *S. faecalis* and N_5-formyl, 5,6,7,8-tetrahydrofolic acid (Leucovorin, Lederle) for *L. citrovorum*. The table also shows the times and places of collection of the different algae.

The *S. faecalis* activity is generally of the order of 0.5–1.0 μg/g dry weight. It is very low in *Ascophyllum nodosum*, 0.07 μg/g, and high in *Chara tomentosa*, 15 μg/g dry weight. The folinic acid activity of the algae is with few exceptions lower than the folic acid activity. In the holdfast of *Laminaria hyperborea* both the folic acid and the folinic acid activity is higher than in the stipe or the frond of this alga. The differences between the folic or folinic acid activity of fertile tips and the rest of the algae are small. The sample of plankton (phytoplankton) possessed no detectable activity for *L. citrovorum*, but contained 0.62 μg folic acid/g dry weight.

The seasonal variation in the total activity for *S. faecalis* in *Alaria esculenta* is shown in Fig. 1. The activity is high during the spring and the summer but low during the autumn. The sudden maximum in January followed by a minimum in February seems to be significant since this effect was obtained also when digestion

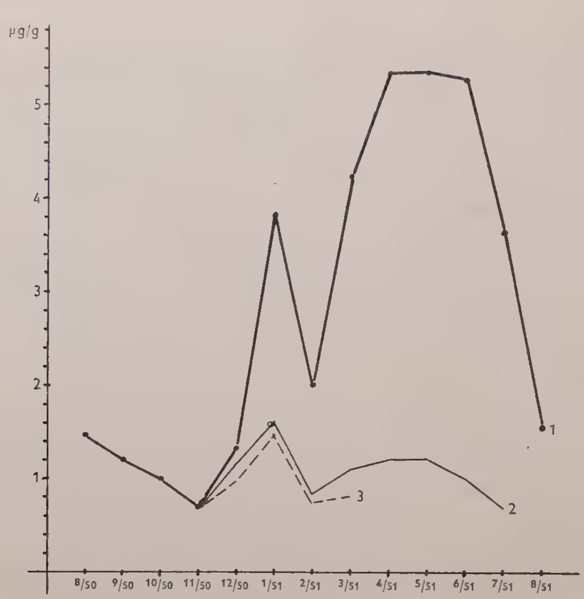

Fig. 1. Seasonal variation in total folic acid activity in *Alaria esculenta* collected at Lofoten, from August 1950 to August 1951. Each figure in the diagram represents an average for at least fifty plants of *Alaria esculenta*. The samples were treated with chicken pancreas homogenate (curve 1), with hog kidney homogenate (curve 2) or autoclaved in water (curve 3).

with hog kidney homogenate or autoclaving in water was used in the extraction procedure (curves 2 and 3). Figure 2, giving the seasonal variation in the total activity for *L. citrovorum*, shows that the activity curve has a sharp maximum in January. In contrast to the observed variation in folic acid activity, no increase in folinic acid activity could be noticed in the spring. Samples treated with hog kidney homogenate or autoclaved in water were also high in folinic acid in January but low in the spring.

The seasonal variations in the total folic acid and folinic acid activities in *Laminaria hyperborea* were also studied. The old and the new blades of the alga were assayed separately. It was observed that the total folic acid activity of both the old and the new blades had maxima in May. In resemblance to the results with *Alaria esculenta* the total folinic acid content of *Laminaria hyperborea* showed no maximum in the spring. No January sample was available. The highest folinic acid value was obtained with a September sample of new blades. Of the old blades the February sample had the highest folinic acid activity. It is therefore clear that the total *L. citrovorum* activity of *Laminaria hyperborea* must have a maximum during the autumn or the winter. The folinic acid activity of the old blades was generally lower than that of the new blades, whereas the folic acid activity of the old blades was considerably higher than that of the new blades. Enough material was not available for a more exhaustive investigation of this alga.

Aqueous extracts of some of the algae in Table I were prepared by autoclaving the algae at 120° C for 15 min. (twice). The pH of each extract was about 6.0. Bioautographic studies of the growth factors for *S. faecalis* present in these solutions revealed

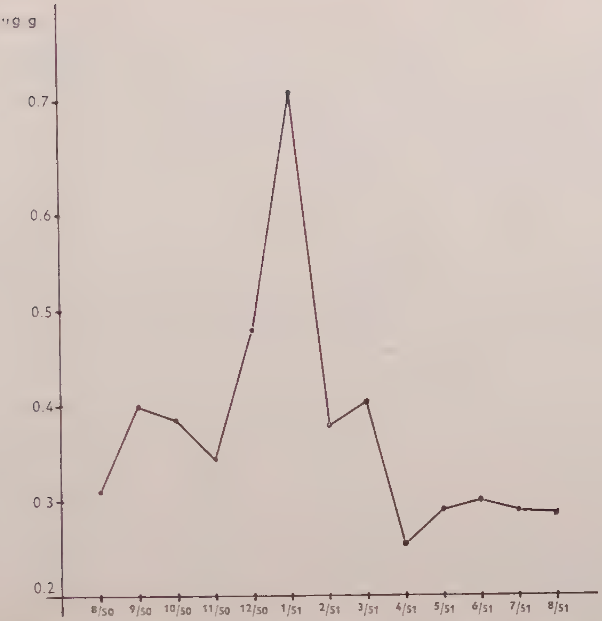

Fig. 2. Seasonal variation in total folinic acid activity in *Alaria esculenta* collected at Lofoten, from August 1950 to August 1951. Each figure in the diagram represents an average for at least fifty plants of *Alaria esculenta*. The algal samples were treated with chicken pancreas homogenate.

a number of active compounds. The spots corresponding to N_{10}-formylpteroic acid, N_{10}-formylfolic acid and N_5-formyl, 5,6,7,8-tetrahydrofolic acid were conspicuously intensive. The high *S. faecalis* activity of the green alga *Chara tomentosa* (15 µg/g) is for instance due almost entirely to N_{10}-formylpteroic acid. Factors with the same R_F-values as the compounds provisionally called Factor I, Factor III and Factor V in a previous paper [1] were also observed in the aqueous extracts. Factor V is active also towards *L. citrovorum* and is probably identical with the synthetic compound believed to be N_5-formyl, 5,6,7,8-tetrahydropteroyldiglutamic acid. The nature of Factor I is still unknown. It appears to be identical with a compound obtained by treatment of pure folinic acid with alkali or with reducing agents [12]. The aqueous extracts of algae seem to contain also N_5-formyl, 5,6,7,8-tetrahydropteroyltriglutamic acid (Factor III). One of the compounds present in the solutions showed the same chromatographical behaviour as the synthetic compound believed to possess this structure and was active towards both *S. faecalis* and *L. citrovorum*.

No striking difference could be observed between the growth factors for *S. faecalis* or *L. citrovorum* present in the fertile tips and in the vegetative thallus. Nor is there any significant difference between the types of growth factors present in algae representing different classes.

Discussion

Table I shows that the total activity for *S. faecalis* and *L. citrovorum* is generally higher in the green and the red algae than in the brown algae. This has been found to hold true also for the total vitamin B_{12}-activity of algae [18] and for the pantothenic acid [19] and the vitamin B_1 [20, 21] contents. The vitamin C [22, 23] and

Table I. Determination of the total activity for *S. faecalis* and *L. citrovorum* in a number of marine algae. [1] = *Himanthalia lorea*, [2] = *Laminaria Cloustonii*. [3] The sample of plankton consisted of Diatoms: *Chaetoceras* (dominating) and *Sceletonema*; and of Dinophyceae (Peridineae) and of a few Copepods. NS = North Sea, ÅS = Åland Sea, AO = Atlantic Ocean.

	Algae	Total activity for		Place and time of collection
		S. faecalis Stand. Folvite μg/g dry weight	*L. citrovorum* Stand. Leucovorin μg/g dry weight	
Rhodophyceae	*Ceramium rubrum*	2.3	0.50	North Berwick, NS 8/52
	Rhodymenia palmata	1.3	0.46	North Berwick, NS 7/52
	Chondrus crispus	0.18	0.12	North Berwick, NS 8/52
	Gigartina stellata	0.66	0.40	North Berwick, NS 8/52
	Porphyra	1.0	0.76	North Berwick, NS 8/52
Phaeophyceae	*Himanthalia elongata*[1]			North Berwick, NS 7/52
	fertile tracts	0.35	0.28	
	vegetative thallus	0.35	0.33	
	Pelvetia canaliculata			North Berwick, NS 8/52
	fertile tracts	0.47	0.34	
	vegetative thallus	0.51	0.29	
	Fucus vesiculosus			North Berwick, NS 7/52
	fertile tracts	0.36	0.24	
	vegetative thallus	0.32	0.22	
	Fucus serratus	0.45	0.50	North Berwick, NS 8/52
	Ascophyllum nodosum	0.07	0.07	North Berwick, NS 8/52
	Alaria esculenta	2.0	0.38	Lofoten, AO 2/51
	Laminaria hyperborea[2]			Dunbar, NS 10/52
	frond	0.31	0.60	
	stipe	0.37	0.70	
	holdfast	0.48	0.72	
	Laminaria digitata	0.47	0.39	North Berwick, NS 7/52
Chlorophyceae	*Chara tomentosa*	15.0	1.1	Öregrund, ÅS 10/52
	Ulva lactuca	1.0	1.1	North Berwick, NS 8/52
	Enteromorpha intestinalis	0.95	0.52	North Berwick, NS 8/52
Heterokontae	*Vaucheria dichotoma*	1.0	0.39	Öregrund, ÅS 10/52
	Plankton[3]	0.62	0	Fiskebäckskil, NS 3/53

the niacin contents [19] appear to be more or less the same in red, brown and green marine algae. *Vaucheria dichotoma* (*Heterokontae*) which contains extremely high amounts of vitamin B_{12} [18] is not unusually rich in folic or folinic acid activity (Table I) or in pantothenic acid or niacin [19]. It is of interest to note that the green alga, *Chara tomentosa*, which has been found to be extraordinarily rich in pantothenic acid [19] is also very high in folic acid activity. The high content of "folic acid" in this alga is not accompanied by a corresponding high content of "folinic acid". The fact that the frond and the stipe of *Laminaria hyperborea* contain less folic and folinic acid activity than the holdfast is in agreement with the findings with vitamin B_{12} [18] and vitamin B_1 [21].

Some of the unknown growth factors for *S. faecalis* and *L. citrovorum* which have been reported to occur in seaweeds [1, 2] and in lichens [11] can now be identified with some certainty. The compound provisionally called Factor III [1] is now believed to be the same as the slowest moving factor for *L. citrovorum* found in the red alga *Furcellaria fastigiata* [2] and the same as the factor with R_F 0.18 found in some lichens [11] and identical with N_5-formyl, 5,6,7,8-tetrahydropteroyl*tri*glutamic acid. The present study has demonstrated the occurrence of this substance in algae. N_5-formyl, 5,6,7,8-tetrahydropteroyl*di*glutamic acid also appears to exist in marine algae. Factor V [1] is probably identical with this last-mentioned compound. The work of DABROWSKA *et al.* [24] and of BOLINDER *et al.* [12] makes it likely that Factor IV [1] is pteroyldiglutamic acid. Factor I [1] has now also been obtained by chemical treatment of folinic acid [12] but the structure of the compound thus obtained is unknown. Enzymatic digestion of various substances also gives rise to a factor with the same R_F-value as Factor I [1, 11, 12].

It is of interest to note that nearly all of the naturally occurring substances, active towards *S. faecalis*, are formylated compounds. The high *S. faecalis* activity of *Chara tomentosa* is, as has already been mentioned, due almost entirely to N_{10}-formylpteroic acid. Other active compounds in this and other algae are N_{10}-formylfolic acid and the mono-, di-, and triglutamates of N_5-formyl, 5,6,7,8-tetrahydropteroic acid.

The different seasonal variations in the folic acid and folinic acid contents in *Alaria esculenta* (Fig. 1.) do not lend support to the assumption that the folic acid and folinic acid groups of factors should always participate in the same biochemical reactions. They rather indicate that the function of "folic acid" may at least in some cases differ from that of "folinic acid". It is noteworthy that the total folic acid content of *Alaria esculenta* is high in the spring when the contents of protein, alginic acid and ash [25] and of vitamin B_{12} [18] have maxima. *Laminaria hyperborea* was also noted to be high in folic acid activity during the spring when protein, alginic acid, ash [25] and vitamin B_{12} [18] are high. The folinic acid activities of *Alaria esculenta* or *Laminaria hyperborea* show no maxima in the spring, but appear to be high in the winter. That the functions of "folic acid" and "folinic acid" may differ in marine algae is further supported by the fact that the ratio of the total folic acid activity to the total folinic acid activity differs widely in different algae (from about 0.5 in the *Laminaria hyperborea* stipe to about 14 in *Chara tomentosa*). The sample of plankton (mainly phytoplankton, see Table I) possessed no detectable activity for *L. citrovorum* but had a *S. faecalis* activity corresponding to 0.62 μg folic acid/g dry weight. Moreover, whereas N_{10}-formylpteroic acid occurs frequently in algae, no evidence has been found for the existence of the corresponding N_5-formylated and reduced form of pteroic acid. The different rate of enzymatic release of the folic acid type of factors as compared to that of the folinic acid type of factors indicates that the two groups of factors may exist in algae in differently bound forms.

SUMMARY

Seventeen marine algae, representing four different classes of algae, and a sample of plankton have been investigated with respect to the growth factors related to folic acid and folinic acid. The total folic acid activity of the algae varied from 0.07 to 15 μg/g dry weight; the total folinic acid activity from 0.07 to 1.1 μg/g dry

weight. Great seasonal variations in the total folic and folinic acid activities have been observed. The difference between the folic acid factors of the various parts of the algae appears to be small. The same holds true for the factors related to folinic acid.

Compounds believed to be the di- and triglutamates of N_5-formyl, 5,6,7,8-tetrahydropteroic acid have been synthesized. Evidence has been obtained for the existence of these compounds (which are active towards both *S. faecalis* and *L. citrovorum*) in aqueous extracts of marine algae. Bioautographic studies have also revealed that the active compounds in aqueous extracts of marine algae are mostly formylated or formylated and reduced derivatives of folic acid.

The results do not indicate that the folic acid group of factors should in all cases possess the same metabolic function as the factors related to folinic acid.

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Studies on the occurrence of amino acids, niacin and pantothenic acid in marine algae

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With 4 figures in the text

The amino acids of marine algae have in the last years been investigated in several laboratories [1–13]. The studies have been concerned not only with the amino acid composition of hydrolysates of the whole plant material but also with investigations on free amino acids and peptides [5–12]. The peptides of marine algae deserve special interest as they may play the same role as the free amino acids of higher plants [12]. Algal peptides have also been considered as the result of restricted photosynthesis due to the low intensity of the light available for most marine algae [5].

Although the results obtained by the different authors have on the whole been in agreement, some differences can be noted. As yet a comparatively small number of seaweeds has been investigated with respect to their amino acid content. No attempt to correlate the occurrence of amino acids with vitamins seems to have been made. It thus appeared justified to carry out further studies on this matter. In the present investigation special attention has been paid to cystine and tryptophane, which have been found by some authors but not by others, as well as to β-alanine, an amino acid not previously reported to be present in seaweed. β-Alanine constitutes part of the pantothenic acid molecule and an attempt has been made to find out whether β-alanine is likely to play some other role in the metabolism of marine algae than that in the pantothenic acid synthesis. The close biochemical relationship between tryptophane and nicotinic acid, which has been observed in a number of cases [14], is one of the reasons for the determination of the niacin content of some seaweeds. Quite apart from these more fundamental problems the determination of these vitamins in seaweed is of interest also from a nutritional point of view.

In a paper on amino acids of marine algae previously published from this laboratory [13] three brown algae (*Phaeophyceae*) of different orders were studied. These algae have been reinvestigated with respect to cystine, tryptophane and β-alanine as well as nicotinic and pantothenic acids. The present paper deals mainly with the amino acids occurring in some red (*Rhodophyceae*) and green (*Chlorophyceae*) algae. For the determination of cystine, tryptophane and β-alanine and of niacin and pantothenic acid, microbiological methods have been employed. Other amino acids reported in this paper have been detected by means of one- or two-dimensional chromatography.

Experimental

Hydrolysis.—Dried and ground algal material (whole plant) was hydrolysed in 6 *N* HCl at 100° C for 24 h. A few samples were hydrolysed under these conditions for both 24 and 48 h. and a comparison of the amino acid distribution in the samples was made. The hydrolysates were evaporated to dryness, dissolved in distilled water and filtered. Alkali hydrolysis was carried out in 14 % $Ba(OH)_2$. The solutions were neutralised with H_2SO_4 and filtered. All hydrolysates were stored at —20° C.

Paper chromatography.—Two-dimensional chromatograms were run in water-saturated phenol (I) and subsequently in a mixture of pyridine (35) *iso*-amylalcohol (35) and water (30) or in (I) and then in water-saturated *n*-butanol containing 2 % acetic acid (II). The spray for these chromatograms consisted of 0.5 % ninhydrin in 96 % ethanol. One way chromatograms were run either on buffered papers according to McFarren [15], on not buffered papers in solvent system (II) or in benzyl-butyl alcohol [15]. For studies on the occurrence of leucine and *iso*-leucine the spray was the same as for the two-dimensional chromatograms. For histidine and tyrosine the spray was prepared from freshly diazotised sulfanilamide (1 %) in a solution of soda [16]. In order to show the presence of proline and hydroxyproline, the chromatograms were sprayed with 0.2 % isatin in a mixture of 96 % *n*-butanol and 4 % acetic acid [16].

All chromatograms were dried in a stream of warm air with a temperature of about 40° C. Whatman No. 1 filter paper was used throughout.

Determination of tryptophane.—Tryptophane was determined microbiologically using *Lactobacillus arabinosus* 17–5 in an agar cup plate method. The Bacto tryptophane assay medium was used with the addition of 1.7 % Bacto agar and 0.5 ml Tween 80/litre medium. The cell suspension had a turbidity corresponding to a Coleman reading of 20 at 5000 Å. 33 ml of this cell suspension was used per litre of medium. Large agar plates which permitted the simultaneous analysis of more than fifty samples were employed. The plates were incubated at 37° C for about 20 h. The standard consisted of DL-tryptophane.

Determination of cystine.—*Lactobacillus arabinosus* 17–5 was employed in an agar cup plate method also for the estimation of cystine. The medium was prepared according to the USP–AOAC collaborative procedure for the determination of niacin [17] but with omission of the cystine and addition of 0.2 mg nicotinic acid/litre medium. To this medium 1.7% bacto agar and 0.5 ml Tween 80/litre medium were added. 33 ml of a cell suspension of *L. arabinosus* having a turbidity of about 20–30 (Coleman reading at 5000 Å) was used per litre medium. As in the case of the tryptophane assay, large agar plates were inoculated with a high number of samples and subsequently incubated at 37° C for about 20 h. L-cystine served as standard. The procedure allows determination of L-cystine at concentrations of about 1 μg/ml and upwards.

Determination of β-alanine.—The medium was that recommended by Atkin *et al.* [18] for determination of pantothenic acid with a strain of *Saccharomyces carlsbergensis* but with the omission of the asparagine as suggested by these authors. *Saccharomyces fragilis* ATCC 2360 was employed as test organism in our case. It was considered desirable to use an agar cup plate method which would allow bioautographic studies and the analysis of a high number of samples with less labour than the tube assay. Bacto agar (1.7 %) was therefore added to the above-mentioned medium. The yeast cells were transferred directly from malt agar slants to 5 ml saline solution and washed once with saline. The turbidity of the cell suspension was adjusted to a Coleman reading of 40 at 5000 Å. 5 ml of this suspension was used per agar plate (150 ml of medium). The plates were incubated overnight at 30° C. Concentrations of β-alanine down to a few tenths of a μg/ml can be determined with this method. The introduction of about 0.5 ml Tween 80/litre medium resulted in sharper edges on the growth zones. This agar cup plate method can also be used for the determination of pantothenic acid.

Determination of nicotinic acid.—For the microbiological determination of nicotinic acid *Lactobacillus arabinosus* 17-5 was employed. The medium consisted of the Bacto niacin assay medium, 1.7 % Bacto agar and 0.5 ml Tween 80/litre medium. The procedure was otherwise identical with that described above for the estimation of tryptophane. Nicotinic acid was used as standard. The nicotinic acid amide is less active than the nicotinic acid in this assay procedure.

Different extraction procedures for nicotinic acid were tried, e.g. water extraction of dried and ground algae, autoclaving in 1 N H_2SO_4 at 120° C for 1 h, boiling in 1 N H_2SO_4 at 100° C for 8 h, in 6 N HCl or in 14 % $Ba(OH)_2$ at 100° C for 24 h. Acid hydrolysis gave higher values than water extraction or hydrolysis in alkali and was therefore used when not otherwise stated. Enzymatic digestion with takadiastase, papain or a homogenate of chicken pancreas was also tried but was without effect.

Determination of pantothenic acid.—Also in this case *Lactobacillus arabinosus* 17-5 served as test organism in a medium consisting of the Bacto pantothenate assay medium, 1.7 % Bacto agar and 0.5 ml Tween 80/litre medium. In all other respects the method followed the description given for the determination of tryptophane. Calcium pantothenate was used as standard.

The extraction of pantothenic acid from the algal material proved to be difficult. Digestion with takadiastase did not give higher values than simple water extraction. The procedure for extraction of pantothenic acid suggested by HARRISON [19], which includes mild treatment with alkali followed by digestion at pH 4.5 with a mixture of papain and takadiastase, caused no increase in the pantothenic acid activity. The enzyme complex of a chicken pancreas homogenate (alone or in combination with takadiastase and/or papain) was not effective in liberating pantothenic acid from seaweeds. Moreover, the pantothenic acid activity of all these enzyme preparations was nearly as high as that of the algae. An extraction procedure in which 1 g of dried and finely ground algal material was autoclaved twice in 10 ml water at 120° C for 20 min. was finally employed. The simultaneous extraction of slimy substances, e.g. alginic acid from the brown and agar-agar from the red seaweeds, made it extremely difficult to work in a strictly quantitative way. It is likely that the values for the pantothenic acid content of seaweeds given in this paper are too low.

Bioautographic separation and identification.—The agar plate method just described for the quantitative determination of some amino acids and vitamins permitted bioautographic studies of these substances. The solvent system used for the chromatographic separation consisted of water-saturated sec.-butanol containing 3 % acetic acid. The chromatograms were dried in a stream of warm air (40° C) and placed on agar plates seeded with the microorganism in question. The agar plates were incubated overnight at the temperatures given above for the respective organisms.

Results

The names of the algae investigated and the places and times of their collection are found in Table II.

In Table I the results of the paper chromatographic identification of the amino acids in the acid hydrolysates are given. Twenty different amino acids have been identified in this way. The R_F-values of some unidentified ninhydrin reacting substances have been collected in the last column of Table I. The intensity of the spots of the different amino acids is indicated by the number of + signs. (+) means that the spot observed was very weak but that the presence in the algae of the corresponding amino acid seems certain. The spot caused by histidine, for instance, was generally very faint on the two-dimensional chromatograms sprayed with ninhydrin, but from the one way chromatograms which were sprayed with diazotized sulfanilamide conclusive evidence was obtained for the presence of this amino acid. As different amino acids are differently sensitive to the ninhydrin reaction [20] the number of + signs in Table I does not directly reflect the relative amounts of the various amino acids. A + + arginine spot is likely to contain more arginine than the alanine present in a + + + α-alanine spot. What Table I shows is, for instance, that the ratio of proline to lysine is greater in *Gigartina stellata* than in *Porphyra.*

α-Amino-*n*-butyric acid seems to occur in small amounts in some algae, e.g. the two species of *Ceramium* investigated. The spot obtained from the acid hydrolysates of the algae believed to contain this amino acid could not be separated from that of pure α-amino-*n*-butyric acid by means of two-dimensional chromatography.

Both leucine and *iso*-leucine are generally present as revealed by chromatography on buffered filter paper [15], but the relative amounts of these two amino acids seem to vary.

Proline, but not hydroxyproline, has been found in all algae investigated.

When very large amounts of hydrolysate of *Ceramium rubrum* were used, a spot occupying the same position on a two-dimensional chromatogram as diiodotyrosine was observed. Another spot having an R_F-value of 0.42 in water saturated phenol and an R_F-value 1.2 times that of phenyl-alanine in the mixture of pyridine, *iso*-amylalcohol and water was also noted. This spot may be due to monoiodotyrosine.

A spot having an R_F-value slightly higher than that of serine in water-saturated phenol and an R_F about 1.5 times that of serine in the mixture of pyridine, *iso*-amylalcohol and water was found in the hydrolysates of some algae. This spot appeared both in acid and alkali hydrolysates of the algae and did not disappear on prolonged hydrolysis. The substance could therefore not be a peptide. It was found to be inseparable from taurine in the two solvent systems just mentioned and also on two-dimensional chromatography with water-saturated phenol as the first and water-saturated *n*-butanol containing 2% acetic acid as the second solvent. For these reasons, taurine is believed to be present in some of the algal hydrolysates. The intensity of the taurine spot made it unlikely that it could be due to impurities of animal origin in the algal material. As can be seen from Table I taurine was found in all red algae investigated except *Ceramium tenuicorne*, and also in the green alga *Ulva lactuca.*

As it was considered possible that taurine might not exist in the living plant but

Table I. Paper chromatographic identification of amino acids in acid hydrolysates of some marine algae. The number of + signs indicates the intensities of the spots, the — signs that no spot due to the amino acid in question was observed. [1] The sample of plankton consisted of Diatoms: *Chaetoceras* (dominating) and *Sceletonema*; of Dinophyceae, (Peridineae), and of a few Copepods. [2] These spot disappeared on prolonged hydrolysis and may therefore have been caused by peptides. [3] Possibly identical with DENT's "fast arginine" [22]. [4] Possibly identical with methionine sulphone. [5] Mono- and diiodotyrosine seem to be present in this alga.

Algae	Amino Acids																		Unidentified substances R_F-phenol
	Gly.	α-Al.	α-Am.-but.	Val.	Leu.	*Iso*-Leu.	Phen. al.	Tyr.	Ser.	Thr.	Meth.	Arg.	Lys.	Prol.	His.	Asp.	Glu.	Tau-rine	
Polysiphonia fastigiata	+++	++	—	++	(+)	(+)	+++	+	++	+	(+)	++	++	++	(+)	++	+++	+	0.32
Ceramium tenuicorne .	+++	+++	+	+++	(+)	(+)	+++	(+)	++	+	+	++	++	+	(+)	++	+++	—	0.16[2], 0.90[3]
Ceramium rubrum . .	+++	+++	(+)	+++	+	(+)	+++	+	+++	++	+	+++	+++	++	(+)	+++	+++	++	0.18[2], 0.60[4, 5]
Chondrus crispus . . .	+++	+++	—	+++	(+)	(+)	+++	+	++	+	+	++	++	++	+	++	+++	+	0.62[4]
Gigartina stellata . . .	+++	++	—	++	+	(+)	+++	+	++	+	+	++	+	+++	(+)	++	+++	(+)	0.25, 0.61[4]
Porphyra	+++	+++	—	++	(+)	(+)	+++	+	++	+	+	+	+	++	(+)	++	+++	+	
Alaria esculenta . . .	+++	+++	—	++	(+)	(+)	++	(+)	++	+	(+)	++	++	++	+	++	+++	—	
Chara tomentosa . . .	+++	+++	—	+++	—	(+)	+++	+	++	+	(+)	+	+	+	(+)	+++	+++	—	0.18[2]
Ulva lactuca	+++	+++	(+)	+++	(+)	(+)	++	+	+++	++	+	++	++	+++	+	++	+++	+	
Vaucheria dichotoma .	+++	+++	—	+++	(+)	+	+++	+	++	++	+	++	+	++	(+)	++	+++	—	0.21, 0.76
Plankton[1]	++	+++	—	+++	(+)	(+)	+++	—	++	+	—	+	+	+	(+)	++	++	—	0.12[2]

may be a post-mortem product, the following experiment was carried out.[1] A sample of *Ceramium rubrum* was collected in a glass jar with sea water and conveyed to the laboratory. One part of the alga (CrA) was immersed in boiling chloroform for a few minutes and dried. The boiling was carried out in order to destroy the algal enzymes, i.e. to prevent post-mortem enzymatic conversions of the algal constituents. Another part of *Ceramium rubrum* (CrB) was dried in air. Both CrA and CrB contained taurine which suggests that taurine may be present in the living alga.

The results of the microbiological determinations of cystine, tryptophane and β-alanine are given in Table II. It can be noted that these amino acids have been found in varying amounts in all algae investigated. The values for cystine and β-alanine are those obtained for the acid hydrolysates whereas tryptophane was determined in the alkali hydrolysates of the algae. It is realised that the fact that the hydrolyses were carried out in the presence of carbohydrates and other substances may have caused considerable destruction of the amino acids. Cystine, tryptophane and β-alanine were also found in the aqueous extracts of the algae but in lower concentrations. The amounts of free β-alanine were significant and generally of the same order of magnitude as those obtained for the acid hydrolysates (see also Fig. 3).

Paper chromatographic separation followed by bioautographic identification of the microbiologically active compounds revealed that the total activity of the hydrolysates was due entirely to cystine, tryptophane and β-alanine, respectively. No interfering substances could be detected. The only compounds in the aqueous extracts which were active in the cystine and the tryptophane tests had the same R_F-values as cystine (R_F 0.41) and tryptophane (R_F 0.27) respectively. When chromatograms of the aqueous extracts were placed on agar plates seeded with *Saccharomyces fragilis* two spots became visible, one due to β-alanine and the other to pantothenic acid. The pantothenic acid spot, however, was very weak compared with the spot caused by β-alanine. On two-dimensional chromatograms sprayed with ninhydrin, the spot due to β-alanine could be observed only when large amounts of hydrolysate were used.

Table II also summarizes the amounts of niacin and pantothenic acid of the seaweeds. The amounts of niacin vary in this table between 1 μg/g for *Ceramium tenuicorne* to 63 μg/g dry weight for *Alaria esculenta*. Considerable amounts of niacin were present also in the aqueous extracts. Bioautography showed that the activity in the aqueous extracts was due mainly to niacin (R_F 0.80). A compound possessing niacin activity and having an R_F-value of 0.15 was observed in some of the aqueous extracts. This compound is thus not identical with niacin or the nicotinic acid amide. In the acid hydrolysates only niacin could be detected.

The pantothenic acid contents show great differences: from less than 0.2 μg/g in some algae and in the sample of plankton tested, up to 12.5 μg/g dry weight in *Chara tomentosa*. The pantothenic acid activity appeared to be caused entirely by pantothenic acid (R_F 0.77) in most extracts. An unidentified growth factor for *L. arabinosus* with an R_F-value of 0.35 in the solvent system employed was found in *Ceramium rubrum*.

Mr. A. Haug, Norsk Institutt for Tang og Tareforskning, Trondheim, Norway, has been kind enough to supply us with monthly samples of the brown alga *Alaria esculenta* collected at Lofoten, Atlantic Ocean. The seasonal variation in cystine, tryptophane, β-alanine, niacin and pantothenic acid has been determined in these samples which are the same as those used by Haug and Jensen in their study of the seasonal variation of carbohydrates, protein and ash in this alga [21]. At least

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[1] The authors are grateful to Dr. W. A. P. Black, Institute of Seaweed Research, Inveresk, Midlothian, Scotland, for the two samples CrA and CrB.

Table II. Microbiological determination of cystine, tryptophane, β-alanine, niacin and pantothenic acid in some marine algae. The — signs mean that no determination was made. [1] See Table I. AO = Atlantic Ocean, NS = North Sea, BS = Baltic Sea (proper), ÅS = Åland Sea.

	Algae	Amino acids			Vitamins		Place and time for collection
		Cystine mg/g	Tryptoph. mg/g	β-Alanine µg/g	Niacin µg/g	Pantothenic Acid µg/g	
Rhodophyceae	*Polysiphonia fastigiata*	0.74	0.30	68	16	2.6	North Berwick, NS 6/52
	Ceramium tenuicorne	0.22	—	42	1	<0.2	Nämdö, BS 10/52
	Ceramium rubrum	0.89	0.35	54	33	2.7	North Berwick, NS 8/52
	Chondrus crispus	0.65	0.52	13	13	1.2	North Berwick, NS 8/52
	Gigartina stellata	0.56	0.68	12	18	1.4	North Berwick, NS 8/52
	Porphyra	0.66	0.17	27	14	2.9	North Berwick, NS 8/52
Phaeophyceae	*Fucus vesiculosus*	0.16	0.20	26	6	<0.2	Simpnäs, ÅS 10/51
	Alaria esculenta	0.08	0.40	36	63	0.18	Lofoten, AO 10/50
	Laminaria saccharina	0.03	0.35	33	23	0.28	Fiskebäckskil, NS 11/51
	Sphacelaria arctica	0.12	0.35	46	11	0.54	Simpnäs, ÅS 10/51
Chlorophyceae	*Chara tomentosa*	0.12	0.35	25	12	12.5	Öregrund, ÅS 10/52
	Ulva lactuca	0.25	0.40	30	20	0.90	North Berwick, NS 8/52
Heterokontae	*Vaucheria dichotoma*	0.34	0.40	48	25	0.90	Öregrund, ÅS 10/52
	Plankton[1]	0.31	—	2	14	<0.2	Fiskebäckskil, NS 3/53

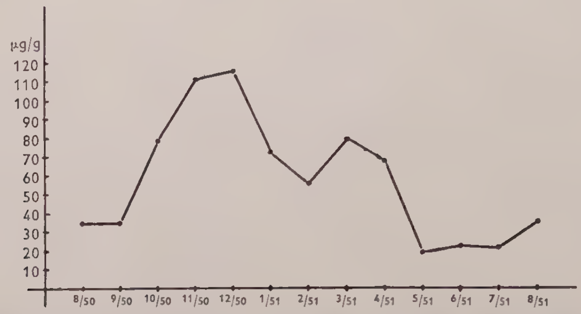

Fig. 1. Seasonal variation in cystine in *Alaria esculenta*, collected at Lofoten from August 1950 to August 1951. The values are those obtained for the acid hydrolysates of the alga.

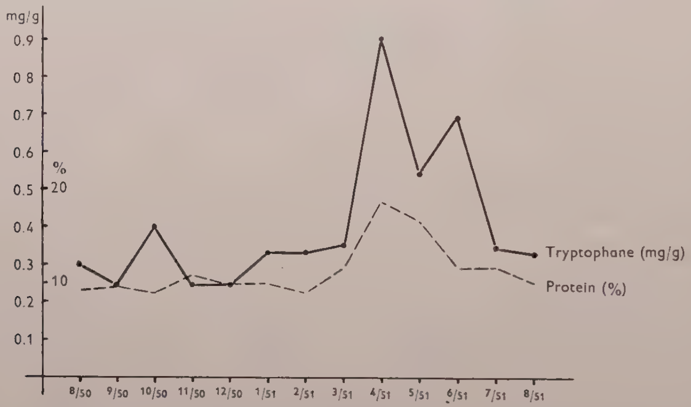

Fig. 2. Seasonal variation in tryptophane in *Alaria esculenta*, collected at Lofoten, from August 1950 to August 1951. The values are those obtained for the alkali hydrolysates of the alga. The curve for protein according to HAUG and JENSEN.

50 plants of *Alaria esculenta* were collected for each monthly sample [21]. Figures 1, 2, 3 and 4 show the variations in cystine, tryptophane, β-alanine, nicotinic acid and pantothenic acid from August 1950 to August 1951.

The curve for the seasonal variation in protein has according to HAUG and JENSEN [21] a maximum in April. It can be seen from Fig. 1 that the content of cystine does not vary in the same way as that of protein (see Fig. 2). The cystine content is highest in November and December with a second maximum in March. The maximum in November and December was shown to be due not to free cystine but to a high cystine content of the protein. Tryptophane (Fig. 2) varies in another way and follows more or less the curve for protein except for the two peaks in October and June. It did not appear as if the content of free tryptophane had a maximum at the same time as the tryptophane of the algal protein. The seasonal variation in β-alanine (Fig. 3) does not resemble the variations either in cystine or in tryptophane. This is true both for the β-alanine content of the acid hydrolysates (curve 1) and that of the aqueous extracts (curve 2). A maximum in total β-alanine occurs in January, but on the whole the variation over the year is slight.

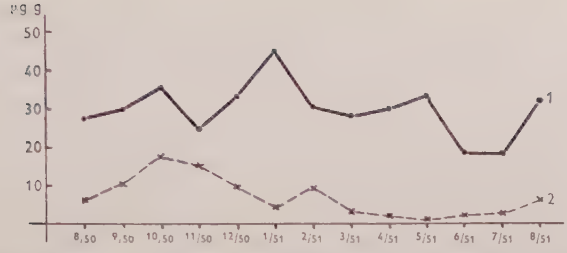

Fig. 3. Seasonal variation in β-alanine in *Alaria esculenta*, collected at Lofoten, from August 1950 to August 1951. Curve 1 gives the β-alanine content of acid hydrolysates, curve 2 the β-alanine content of aqueous extracts of the different algal samples.

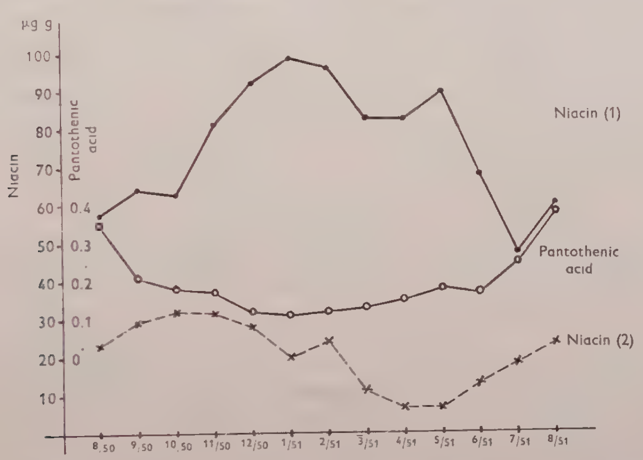

Fig. 4. Seasonal variation in nicotinic acid and pantothenic acid in *Alaria esculenta* collected at Lofoten, from August 1950 to August 1951. The two curves for niacin represent the activity in acid hydrolysates (curve 1) and in aqueous extracts (curve 2) of the different algal samples respectively.

Figure 4 shows the seasonal variations in niacin and in pantothenic acid. The niacin content of the acid hydrolysates is high during the winter months and also in May. The pantothenic acid content of *Alaria esculenta* appears to have a maximum in August and a minimum in January.

The different samples of *Alaria esculenta* were submitted to bioautographic studies. It was observed that the values for cystine, tryptophane, β-alanine and niacin of the hydrolysates could be attributed only to these compounds, no other compounds active for the respective test organisms being detected. The same was true for the pantothenic acid. The niacin content of the aqueous extracts was observed to be due both to niacin and to the active compound with R_F 0.15 mentioned above.

Discussion

The results of the paper chromatographic identification of amino acids in the seaweeds investigated agree on the whole with those reported in a previous paper [13] from this laboratory. We then failed to detect tryptophane in the three marine algae *Fucus vesiculosus*, *Laminaria saccharina*, and *Sphacelaria arctica*. SMITH and E. G. YOUNG [9] have reported tryptophane to be absent from the protein of *Fucus vesiculosus*. From the present study it is evident, however, that tryptophane is present in these algae as well as in other red, brown and green algae. CHANNING and C. T. YOUNG [11] have found tryptophane in *Pelvetia canaliculata* and in *Rhodymenia palmata*. It can be concluded that tryptophane occurs in most marine algae both in the free and in the bound form, although the amount varies considerably over the year (Fig. 2).

SMITH and E. G. YOUNG [8] and CHANNING and G. T. YOUNG [11] have reported that cystine is not present in acid hydrolysates of various marine algae. This amino acid had previously been claimed to occur in some brown algae [13] and has recently been found in marine algae also by COULSON [12]. The microbiological determination of this amino acid in the present study provides convincing evidence for its existence in a number of different seaweeds. The seasonal variation in the cystine content of *Alaria esculenta* does not seem to run parallel to the variation in cystine in *L. Cloustonii* [12]. In the latter alga, COULSON [12] found that the cystine (cysteic acid) content increases from January to May and remains high over the summer. Cystine is said to be absent in September and low in October, November and December. In *Alaria esculenta* the cystine is high in October, November and December, shows a minimum in May and is low during the summer months (Fig. 1). The content of free cystine in *Alaria esculenta*, which was less than one-tenth of the total cystine content, appeared to be high in the autumn and showed no maximum in May.

The qualitative difference in the amino acid composition of the algae investigated is small in spite of the fact that the algae represent four different classes. Tyrosine and methionine are present in all algae, except in the sample of plankton (phytoplankton) which, however, was examined less exhaustively than the other algae. All the essential amino acids have been found.

A comparison of the curves for the seasonal variations in tryptophane (Fig. 2) and in niacin (Fig. 4) shows that the maxima and minima of the two curves do not coincide. There is rather a tendency for the total niacin content to be high when the tryptophane content is low, and vice versa. Also the niacin of the aqueous extracts tends to be low, e.g. in the spring, when tryptophane is high. The curves for β-alanine (Fig. 3) and for pantothenic acid (Fig. 4) show a similar relationship: the total content of β-alanine has a maximum in January when the pantothenic acid content has a minimum. The concentrations of β-alanine and niacin of the aqueous extracts varied in a similar way, whereas the pantothenic acid content of the same extracts showed a completely different variation.

The niacin activity of the water extracts of *Alaria esculenta* was so low compared with the niacin activity of the acid hydrolysates that one must assume the existence of compounds which are inactive in the microbiological test but are converted to niacin on acid hydrolysis. This was observed also for some of the other algae investigated. Such substances, e.g. trigonelline, are known to occur in other plant material, for instance in cereals [23].

It has already been mentioned that significant amounts of free β-alanine are present in some marine algae. The β-alanine occurring in bound form is, however, so high that it is improbable that all of it should exist in the form of pantothenic acid, although it is likely that the values given above for the content of pantothenic acid are too low. β-Alanine must therefore play some other role in the metabolism of marine algae.

In fact, the concentration of β-alanine is so high that it could well constitute part of a protein molecule. NIELSEN *et al.* [24] found appreciable amounts of β-alanine in the yeast *Rhodotorula gracilis* which had been cultivated in a nitrogen-rich medium. The *Lactobacillus bulgaricus* factor (LBF-1A), which is closely related to pantothenic acid and whose chemical nature has been elucidated recently, is a derivative of β-alanine [25]. Part of the total β-alanine content may have been formed by decarboxylation of aspartic acid. Further studies are required to account for the β-alanine content of marine algae.

The existence of taurine in some marine algae is interesting as little seems to be known about the occurrence of this acid in plant material. The experimental data presented suggest that taurine may be present in the living alga. One could assume that the taurine had been formed from cysteine through decarboxylation and oxidation of the SH-group of cysteine to the SO_3H-group of taurine but this does not appear to be the case. The taurine of *Ceramium rubrum* was found to occur only in free form.

Marine algae provide a good source of niacin. The pantothenic acid content of seaweed appears to be low.

SUMMARY

The occurrence of amino acids, niacin and pantothenic acid has been investigated in a number of marine algae by means of paper chromatography and microbiological methods. The seasonal variations in cystine, tryptophane, β-alanine, niacin, and pantothenic acid in *Alaria esculenta* have been studied.

Twenty-two different amino acids have been found, among them glycine, α-alanine, β-alanine, α-amino-*n*-butyric acid, valine, leucine, *iso*-leucine, phenylalanine, tyrosine, serine, threonine, cystine, methionine, lysine, arginine, tryptophane, proline, histidine, aspartic acid, and glutamic acid. As well as these twenty amino acids, mono- and diiodotyrosine have been observed in the red alga *Ceramium rubrum*. Taurine has also been found and seems to occur in free form in the living alga. Cystine, tryptophane and β-alanine occur both in the free and the bound form. No significant qualitative variation in the amino acid composition of the different algae was noted. The experiments show that the composition of the algal protein undergoes great seasonal variations.

The niacin content of the algae investigated was generally quite high and varied from about 1 μg/g in *Ceramium tenuicorne* to about 99 μg/g dry weight in *Alaria esculenta* (January sample). The content of pantothenic acid was low with the exception of *Chara tomentosa* in which 12.5 μg/g dry weight was found. Both the niacin and the pantothenic acid contents show considerable seasonal variations.

Acknowledgements

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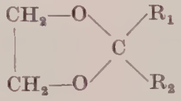
Communicated 3 June 1953 by H. v. EULER and KARL MYRBÄCK

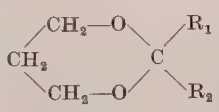
A kinetic study of the acid hydrolysis of cyclic acetals

By OLOF CEDER

With 2 figures in the text

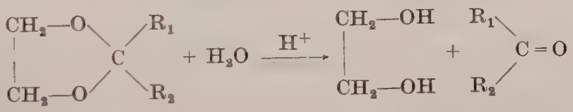
The present investigation is a reaction kinetic study of the acid hydrolysis of cyclic acetals of the type:





where R_1 or R_2 or both are aromatic radicals.

When hydrolysed a diol and an oxo-compound are formed:



The present communication deals mainly with the effect of R_1 and R_2 upon

1. the stability in acid medium,
2. the energy of activation,
3. the frequency factor

and the mechanism of the reaction.

Only two papers on the stability in acid medium where the substituents R = alkyls have been published (1, 2).

The reaction has been followed by measuring the formation rate of the oxo-compound in a spectrophotometer and also by titration.

Experimental

Preparation of the acetals.

As starting materials practical ethylene glycol which was refractionated (b.p. 92–94/14 mm and $n_D^{20} = 1{,}4322$) and practical trimethylene glycol (b.p. 118–120/14 mm and $n_D^{20} = 1{,}4391$) were used. The benzaldehyde, cinnamic aldehyde, acetophenone and benzophenone were of quality "Merck DAB 6" and the *p*-toluene sulphonic acid a pro analysi quality from Schering-Kahlbaum.

The acetals were prepared according to the excellent method of azeotrope distillation of SALMI (3) and KÜHN (4) with a slight modification:

Equivalent quantities of the diol and oxo-compound (about 15 g) were dissolved in 175 ml benzene and 0.1 g *p*-toluene sulphonic acid was added. The distillation was carried out in an ordinary Soxhlet extraction apparatus and the granular calcium chloride as dehydrating agent was placed in the thimble and this was replaced twice during the distillation. The time of extraction varied from six hours to four days (see table 1). After the extraction was complete the benzene was removed by distillation and the residue fractionated under reduced pressure. This product was usually purified either by further refractionation or by recrystallisation from petroleum ether.

The physical properties of these compounds are listed in table 1. The melting and boiling points are uncorrected.

Table 1.

Compound	Extr. time.	B. p.	M. p.	n_D^{20}
1,3-dioxolanes:		a)	a)	a)
1,3-dioxolan	b)	74.0° 73–75° (6)		
2-phenyl	1.6 d	109–10°/14 101°/10 (7)		1.5267 1.52696 (7)
2-methyl-2-phenyl	2	100°/14 102.0–103.5°	60–62° 59.5° (8)	
2,2-diphenyl	4	170–71°/11 168°/10 (7)	55–56°	
2-styryl	1.5	148–49°/13 137.2–138.8°/4 (9)	31–32° 34° (10)	1.5648 1.56536 (9)
1,3-dioxanes:				
2,2-dimethyl	3	124–26°/760 124.0–124.2°/758 (11)		1.4190 1.42007 (11)
2-phenyl	6 h	122–24°/11	49.5–50.0° 49–51° (12)	
2-methyl-2-phenyl	2 d	105–10°/14 103.5–103.9°/12 (8)	48–49° 57.5° (8) c)	
2,2-diphenyl	2	172–74°/12	111.5–12.5° d)	
2-styryl	1	165°/12 143.3°/2.5 (7)		1.5602 1.56111 (7)

a) the values on the second line are reported from the literature
b) prepared by the phosphoric acid method of VERLEY (5)
c) probably an error-reading of 10°
d) recrystallized in the following way: 50 mg was dissolved in 50 ml 1,4-dioxan and was made up to 1000 ml with water. After some hours a pure crystalline product was obtained.

Analytical methods.

When hydrolysed an aldehyde(ketone) and a diol are formed. It is most convenient to determine the aldehyde concentration and this determination was done in the following investigation.

1,3-dioxolan forms formaldehyde and this was determined in samples drawn out at different times by the sulfite method of LEMME (13):

To 20 ml of the sample five drops of thymol phthalein (0.1 % in 80 % ethanol) was added and the hydrolysis was interrupted by neutralizing with 1 N NaOH. After a careful neutralization (to a faint blue colour of the indicator) with 0.1 N HCl 20 ml 1 molar Na_2SO_3 (neutral against thymol phtalein) was added:

$$CH_2O + Na_2SO_3 + H_2O \rightarrow CH_2O(NaHSO_3) + NaOH$$

and the liberated NaOH titrated with 0.1 N HCl.

2,2-dimethyl-1,3-dioxan forms aceton which was determined by the iodoform method of MESSINGER (14) as modified by ELSNER (15):

To a sample of 5 ml, which was made alkaline with 15 ml 1 N NaOH, 10–40 ml 0.1 N iodine was added, and after shaking for five minutes and acidifying with 20 ml 0.5 molar H_2SO_4 the iodine was titrated against 0.1 N $Na_2S_2O_3$ using starch as indicator

$$(CH_3)_2CO + 3\,J_2 + 4\,KOH \rightarrow CH_3J + CH_3COOK + 3\,H_2O\,.$$

For the remaining acetals it was not possible to use titration methods, since they are too difficult to dissolve in water to form a quantity of the oxo-compound large enough to determine and also they are too easily hydrolysed. All the aromatic oxo-compounds have, however, a very strong absorption in ultraviolet region (16). The carbonyl group has a maximum absorption at 2450–2920 Å and molar extinction coefficients of 10–20 000 make it possible to determine quantities as low as $5 \cdot 10^{-6}$ moles per liter by measuring the absorption. The measurements were carried out in a "Mod. D. U. Beckman Quartzspectrophotometer" with a hydrogen lamp and 1 cm quartz cells at wavelengths given in table 2.

Table 2.

Benzaldehyde	Acetophenone	Benzophenone	Cinnamic aldehyde
2495	2455	2600	2920 Å

The absorption spectra of the cyclic acetals show that no one of them with the exception of the cinnamic aldehyde acetals have any maximum above 2100 Å which is the lower limit of the apparatus. The cinnamic aldehyde acetals, however, have strong maxima at about 2500 Å due to their ethylenic double bond. The ethylene acetal has its maximum at 2520 Å and the trimethylene acetal at 2500 Å. From figure 1 it appears that the absorption curves of the acetal and the aldehyde overlap each other a little. As long as the aldehyde concentration is low one can neglect its interfering absorption upon the acetal as the molar extinction coefficients of the acetals are of the same magnitude as that of the cinnamic aldehyde.

This permits the measurement of the two components in the system and also the calculation of the hydrolysis constants in two ways for the cinnamic aldehyde acetals.

The kinetic measurements were carried out in the following way:

The acetal was weighed out in a quantity which after complete hydrolysis gave an extinction of about 0.5. This quantity was dissolved in 3 ml 1,4-dioxan and the solution was made up to 1 liter with water. (The benzophenone acetals, however, required 50 ml 1,4-dioxan to remain in solution after the addition of water.) 5 ml of this solution was made up to 25 ml with hydrochloric acid of a suitable concentration, transferred into a cuvette and the extinction was measured at different times against a blank containing the same quantity of 1,4-dioxan using a slit width of 1.0.

The H^+-concentration was chosen so that the period of half-life was 10–15 minutes. During the hydrolysis the temperature was kept constant ($\pm 0.1°$) with thermostat water which was lead through the thermospacers. The temperature was controlled with a thermometer placed in the cuvette rack. Before the beginning of the experiments the solutions were placed in the thermostat until they assumed constant temperature.

The catalyzing hydrochloric acid was diluted to a suitable concentration from a 0.1000 N stock solution. This value of c_{H^+} was used for the calculation of the hydrolysis constant.

After each run the pH was checked in a potentiometer, "Type PHM 3G Radiometer København" with a saturated solution of potassium bitartrate as reference buffer.

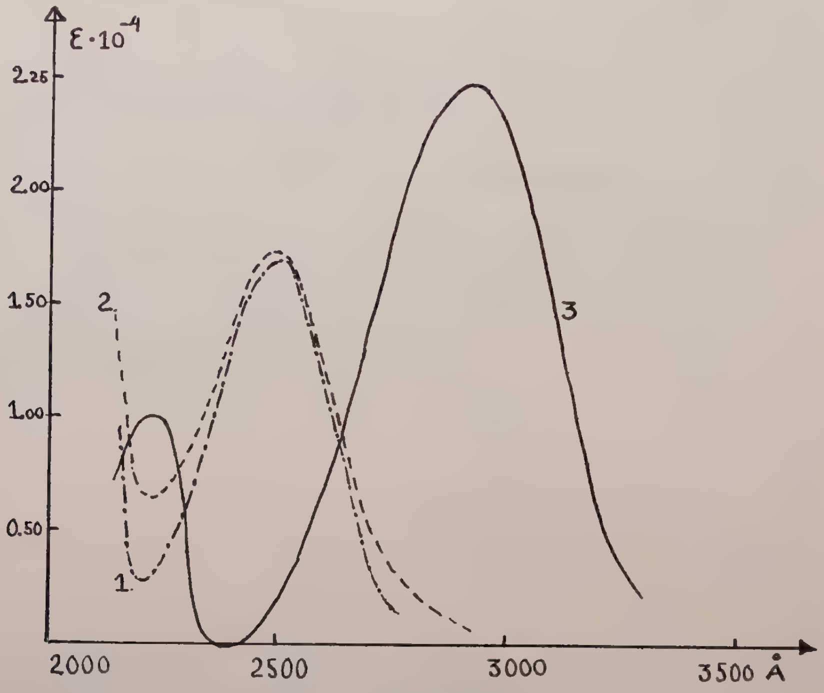


Fig. 1. Curve 1: ethylene acetal.
» 2: trimethylene acetal.
» 3: cinnamic aldehyde.

Calculations

The hydrolysis, catalyzed by acids, has been proved earlier (1, 2) to be monomolecular and hence the first order equation can be applied:

$$K_s[\mathrm{H}^+] = \frac{2.303}{t-t_0} \cdot {}^{10}\log\frac{c_0}{c} \tag{1}$$

where c_0 is the initial, c the concentration at the time t and K_s the disintegration constant of the acetal.

For 1,3-dioxolan and 2,2-dimethyl-1,3-dioxan c was calculated for every t from the titration values, but in the other cases the measured extinction, E, was used directly.

From Beer's law we have for the oxo-compound:

$$E = \varepsilon \cdot c \cdot l$$

where ε is the molar extinction, c the concentration and l the thickness of the absorption layer. From this we have:

$$c = \frac{E}{\varepsilon \cdot l}$$

but

$$c_{\text{acetal}} = c_\infty - c$$

where c_∞ is the concentration of the oxo-compound when $t = \infty$. Hence when the acetal is totally hydrolyzed it satisfies the following equation:

$$c_{\text{acetal}} = \frac{E_\infty - E}{\varepsilon} \quad \text{and} \quad c_{0\ \text{acetal}} = \frac{E_\infty}{\varepsilon}.$$

If this is put into (1) we obtain:

$$K_s[\mathrm{H}^+] = \frac{2.303}{t - t_0} \cdot {}^{10}\log \frac{E_\infty}{E_\infty - E}$$

and

$${}^{10}\log\,(E_\infty - E) = -\frac{K_s[\mathrm{H}^+]}{2.303} \cdot t + \text{const.}$$

If we plot log $(E_\infty - E)$ against t we get a straight line and from its slope K_s can be calculated.

By the numerical computation of K_s the average value was calculated from the following formula for repeated observations:

$$K_s = \frac{2.303}{[\mathrm{H}^+]} \cdot \frac{\sum\limits_{p=1}^{n} {}^{10}\log\,(E_\infty - E)_p - \sum\limits_{q=n+1}^{2n} {}^{10}\log\,(E_\infty - E)_q}{\sum\limits_{r=1}^{n} t_r - \sum\limits_{s=n+1}^{2n} t_s}$$

where $2n$ is the number of observations.

In this way the velocity constants were determined by measuring the velocity of formation of the oxo-compound from its absorption.

By measuring the change of the absorption of the cinnamic aldehyde acetals with time one can determine the velocity constant, too, and thus we have:

$${}^{10}\log\,E = -\frac{K_s[\mathrm{H}^+]}{2.303} \cdot t + \text{const.}$$

If we plot log E against t we get a straight line from which K_s can be calculated. Gradually, however, the values deviate from the line depending on the interfering absorption of the aldehyde. The numerical calculation of K_s was carried out in the same manner as in the previous case. The values were used only so long as they agreed with the straight line. Both the change of the aldehyde- and the acetal-concentration were measured in the same sample, and this facilitates the calculation of the velocity constants in two different ways for every cinnamic aldehyde acetal.

Results

As an example of the measurements the hydrolysis of 2-phenyl-1,3-dioxolan is taken. See table 3.

As an example of the cinnamic aldehyde acetals the hydrolysis of 2-styryl-1,3-dioxolan is taken. See tables 4 and 5 and figure 2.

Table 3.

2-phenyl-1,3-dioxolan.

Quantity: 32.8 mg
c_{H^+}: 0.20 mC
pH: 3.11

Temp.: 20.0° C
Wavelength: 2495 Å
Slit: 1.0

Min	E	$E_\infty - E$	$\log (E_\infty - E)$
1	0.112	0.535	0.728 − 1
2	142	505	703
3	168	479	680
4	193	454	657
6	240	407	610
8	283	364	561
10	321	326	513
12	360	287	458
14	384	263	420
16	411	236	373
20	457	190	279
25	501	146	164
30	539	108	33
36	570	77	887 − 2
42	588	59	771
45	600	47	672
50	612	35	544
∞	647		

From these values we get $K_s = 68.6 \text{ min}^{-1}$.

Table 4.

2-styryl-1,3-dioxolan.

Quantity: 22.4 mg
c_{H^+}: 0.08 mC
pH: 4.13

Temp.: 20.0° C
Wavelength: 2520 Å
Slit: 1.0

Min	E	$\log E$
1	0.335	0.525 − 1
3	304	483
5	273	436
7	249	396
9	228	358
11	208	318
13	195	290
15	179	253
17	168	225
19	159	201
23	144	158
29	127	104
35	119	76
41	112	49
49	108	33
∞	100	000

From the eight first values we get $K_s = 559 \text{ min}^{-1}$.

Table 5.

2-styryl-1,3-dioxolan.

Quantity: 22.4 mg
c_{H^+}: 0.08 mC
pH: 4.13

Temp.: 20.0° C
Wavelength: 2920 Å
Slit: 1.0

Min	E	$E_\infty - E$	log $(E_\infty - E)$
2	0.293	0.350	0.544 − 1
4	342	301	479
6	384	259	413
8	422	221	344
10	452	191	281
12	479	164	215
14	502	141	149
16	523	120	79
18	541	102	9
20	555	88	945 − 2
24	579	64	806
28	597	46	663
34	612	31	491
40	623	20	301
50	634	9	954 − 3
∞	643		

From these values we get $K_s = 954$ min^{-1}.

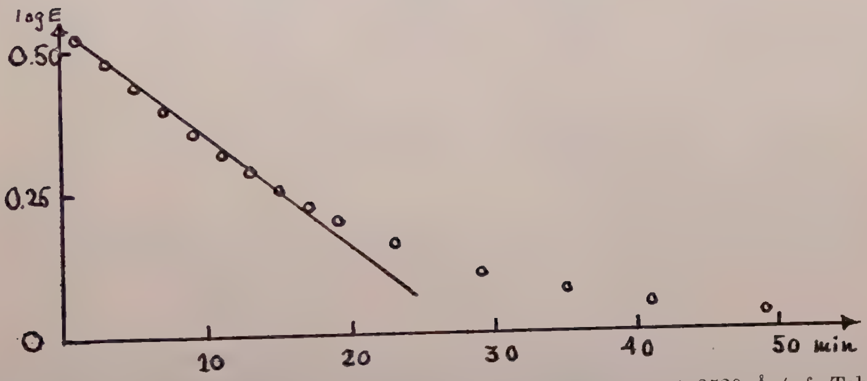


Fig. 2. $^{10}\log\ E = f(t)$ for 2-styryl-1,3-dioxolan from measurements at 2520 Å (c.f. Table 4).

The results of the measurements where the energy of activation and the frequency factor has been calculated from the velocity constants at 20° and 30° C by the equation of Arrhenius

$$K_s = s \cdot e \frac{-E_a}{RT} \qquad (K_s \text{ in sec}^{-1})$$

are summarized in table 6.

Table 6.

Compound	K_s 20° C	K_s 30° C	E_a	s
1,3-dioxolan	$1.34 \cdot 10^{-4}$	$5.91 \cdot 10^{-4}$	27.1	$1.6 \cdot 10^{14}$
2,2-dimethyl-1,3-dioxan	101	322	19.8	$1.8 \cdot 10^{15}$
2-phenyl-1,3-dioxolan	68.6	159	14.8	$1.3 \cdot 10^{11}$
2-phenyl-1,3-dioxan	14.2	46.2	20.9	$8.6 \cdot 10^{14}$
2-methyl-2-phenyl-1,3-dioxan	16.7	44.8	17.5	$2.9 \cdot 10^{12}$
2-methyl-2-phenyl-1,3-dioxan	88.4	265	19.3	$3.8 \cdot 10^{14}$
2,2-diphenyl-1,3-dioxolan	1.85	4.92	17.2	$2.1 \cdot 10^{11}$
2,2-diphenyl-1,3-dioxan	7.20	19.3	17.4	$1.0 \cdot 10^{12}$
2-styryl-1,3-dioxolan	954	1955	12.7	$4.4 \cdot 10^{10}$
2-styryl-1,3-dioxan	281	647	14.7	$4.2 \cdot 10^{11}$

In table 6 K_s is reported in min^{-1}, the energy of activation in kcal per mol and the frequency factor, s, in sec^{-1}.

The results from the measurements on the cinnamic aldehyde acetals are summarized in table 7.

Table 7.

Compound	Wavelength	K_s 20° C	K_s 30° C	E_a	s
Ethylene acetal	2520 Å	559	1129	12.4	$1.7 \cdot 10^{10}$
	2920	954	1955	12.7	$4.4 \cdot 10^{10}$
Trimethylene acetal	2500	212	453	13.4	$3.4 \cdot 10^{10}$
	2920	281	647	14.7	$4.2 \cdot 10^{11}$

Discussion of the kinetic measurements

Stability towards acid hydrolysis.

From the present values and those of LEUTNER (1, 2) we get the following series of oxo-compounds according to the stability of their cyclic acetals:

5-membered.	*6-membered.*
formaldehyde	formaldehyde
acetaldehyde	acetaldehyde
benzophenone	benzophenone
acetone	benzaldehyde
acetophenone	acetophenone
benzaldehyde	acetone
cinnamic aldehyde	cinnamic aldehyde

We can observe that there is the same order for both 5- and 6-membered with the exception of acetone and benzaldehyde which have changed their places. From the K_s-values of table 6 (see also LEUTNER (1, 2)) we observe that those of the formals are of a quite different magnitude than the other ones. The comparison between

5- and 6-membered acetals confirms the synthetic relation that aldehydes prefer to form 6-rings while ketones prefer 5-rings.

The energy of activation and the frequency factor.

It is known (17) that the hydrolysis of an acetal is characterized by a high temperature coefficient of about 4 which gives an energy of activation of about 25 kcal/mol, and a large frequency factor. This was found to be the case with the open acetals but we have no information about the cyclic ones. In accordance with this we would expect to find values of the same magnitude here. This was, however, found to be different.

If we first consider 1,3-dioxolan, a formal, we find $E_a = 27.1$ kcal/mol and $s = 1.6 \cdot 10^{14}$; 2,2-dimethyl-1,3-dioxan, a ketal, also gives very large values: 19.8 kcal/mol and $1.9 \cdot 10^{15}$. These are of the same magnitude as those of the corresponding open formals and ketals. Therefore we conclude that the ring closure has very little effect upon the constants.

The aromatic acetals can be compared more systematically when both 5- and 6-membered rings are examined. If we at first consider the energy of activation we find it in each case larger for the dioxanes (6-membered) than for the dioxolanes (5-membered). Benzaldehyde gives a considerable difference: 20.9 resp. 14.7 kcal/mol and the cinnamic aldehyde the lowest values with a small difference: 14.7 resp. 12.7 kcal/mol. Benzophenone has practically the same value for both and acetophenone shows a difference of 2 kcal/mol. From these we conclude that the energy of activation is more dependent on the nature of the oxo-compound than of the acetal character.

If we compare the frequency factors we observe the same relation: s is always larger for 6- than for 5-membered acetals. We have the ratio

$$s_{\text{6-membered}} : s_{\text{5-membered}}$$

Benzaldehyde	Acetophenone	Benzophenone	Cinnamic aldehyde
6620	131	4.8	9.6

The ratio

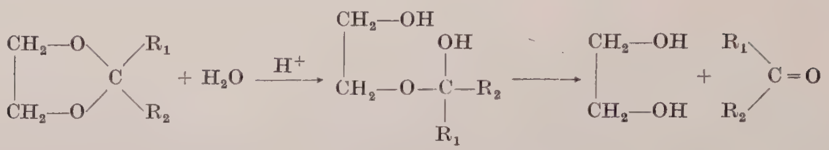
$$s_{\text{benzaldehyde}} : s_{\text{acetophenone}} : s_{\text{benzophenone}} : s_{\text{cinnamic aldehyde}}$$

5-membered	3.0	66	4.8	1
6- »	2050	905	2.4	1

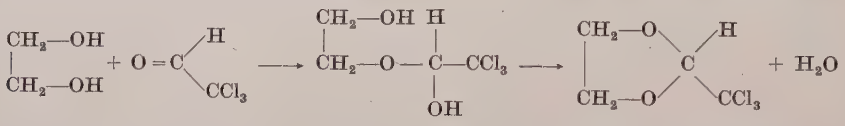
gives a relation between the substituents and the frequency factor. In the 5-membered rings we have a pronounced difference between acetophenone on one hand and the rest on the other and in the 6-membered benzaldehyde and acetophenone differ from benzophenone and cinnamic aldehyde.

Discussion of the mechanism of the hydrolysis.

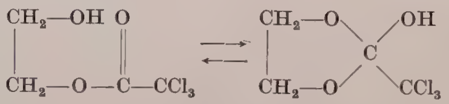
In the formation of the open aliphatic acetals it has been shown by measuring the change of the refraction index (18, 19) and of the volume with a dilatometer (19) that there is a two-step reaction. In the first step a semi-acetal forms and in the second a "Vollacetal". Attempts to isolate the semi-acetal have always proved to be unsuccessful. The cyclic acetals certainly follow the same scheme (20):



A proof for this is the reaction between ethylene glycol and chloral (20) which takes place in two steps and the intermediate, hydroxy-tri-chloro-ethylidene-glycol, is stable and crystalline. When sulfuric acid is added the cyclic acetal is formed:

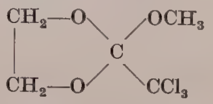


Moreover it has been proved (21, 22) that tri-chloroacetyl-glycol takes part in the following tautomeric equilibrium:



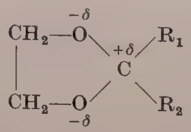
I II

where II can be regarded as a 2-hydroxy-1.3-dioxolan. The position of the equilibrium has been approximately determined from the molar refraction. With diazomethane only the following cyclic compound is formed:



From this the authors assume the existence of the cyclo-tautomer.

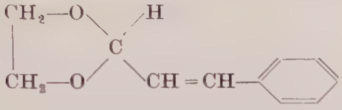
From measurements of the dipole moment of cyclic acetals (23) the following distribution of the electrical charge has been proposed:



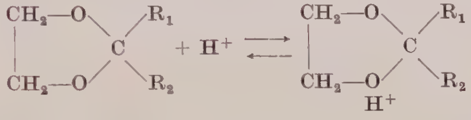
If R_1 and R_2 are strongly electron repelling groups the negative charge at the oxygen atoms will be less than if they are strongly electron attracting. As the mechanism consists of an attack of the hydronium ions on the negative oxygen atoms (24) the acetals with electron repelling substituents ought to be more stable than those with electron attracting substituents. The experimental results are in agreement with this theory. By the comparison of a formal (R = H) and a ketal where R is a phenyl group we find constants of quite different order and the formal the most stable. The same relation exists when only one of the substituents is a phenyl group.

It is especially interesting when one or both are electron attracting substituents. The tri-chloro-acetyl-glycol and chloral acetal can be cited as examples.

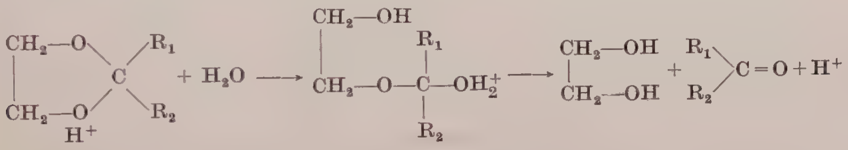
Further we observe that the velocity of hydrolysis is highly increased when as in the cinnamic aldehyde acetals a double bound conjugated with the phenyl group is present:



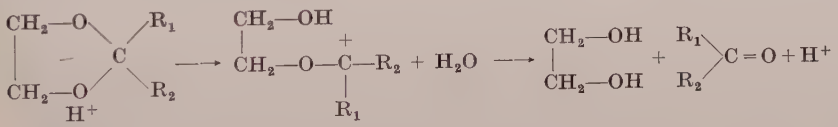
There are consequently several experimental facts which indicate this distribution of the charge and mechanism. The first step of the reaction would therefore consist in a rapid addition of a proton with the formation of an oxonium ion:



For the second and rate-determining step, the formation of the oxo-compound two mechanisms are possible. On one hand the reaction between the oxonium ion and water with the formation of a semi-acetal which decomposes:



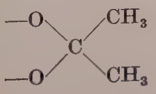
and on the other the splitting of the oxonium ion under the formation of a carbonium ion which reacts with one mol water:

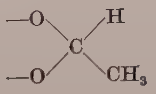


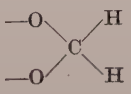
From the present measurements the carbonium ion mechanism is preferred for the following reason. The velocity of hydrolysis would in this case be proportional to the concentration of the carbonium ion. The tendency to form a carbonium ion increases for the carbon atom in the following order

tertiary > secondary > primary (25).

Considering some of these compounds we have for 5-membered:

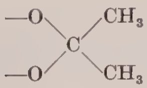


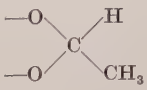


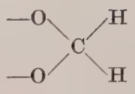


K_s 8.74 0.78 $2 \cdot 10^{-4}$ (min^{-1})

and for 6-membered:







K_S 338 0.13 $5 \cdot 10^{-5}$ (min^{-1})

which is in agreement with the theory. Further a benzyl and an allyl carbonium ion is formed especially easily (25) and in accordance with this the aromatic acetals are much less stable than the aliphatic ones.

Measurements on the cinnamic aldehyde acetals.

From these measurements both the disintegration constant of the acetal, K_1, and the formation constant of the oxo-compound, K_2, were determined. The two methods give, however, values which are not in agreement and their difference lies considerably out of the range of experimental error.

The ratio K_2/K_1 is for the 6-membered 1.7 and for the 5-membered acetal 1.3.

The original intention with these trials was to obtain an experimental evidence for a two-step reaction with a different velocity in each step. The difference is, however, too slight to use the results for a mathematical analysis of the reaction. More convenient compounds for this purpose are probably the cyclic acetals of chloral.

I am very much indebted to Prof. Dr. HANS VON EULER for suggesting this problem to me, for the facilities he offered me in his laboratory and for his neverfailing help and encouragement. I am also very grateful to Prof. Dr. KARL MYRBÄCK for his keen interest during the course of this investigation.

SUMMARY

1. The acid hydrolysis of ten cyclic acetals, aromatic derivatives of 1.3-dioxolan and 1.3-dioxoan, has been studied. The reaction has been followed by measuring the ultraviolet absorption in a spectrophotometer.

2. The effect of different substituents upon the velocity of the hydrolysis, the energy of activation and the frequency factor has been discussed.

3. A two-step reaction including a carbonium ion mechanism is proposed.

Institute for Organic Chemistry and Biochemistry, University of Stockholm.

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Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

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Optically active higher aliphatic compounds. XII

trans-2,5D-Dimethyl-$\Delta^{2:3}$-heneicosenoic acid and *trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid

By STINA STÄLLBERG-STENHAGEN

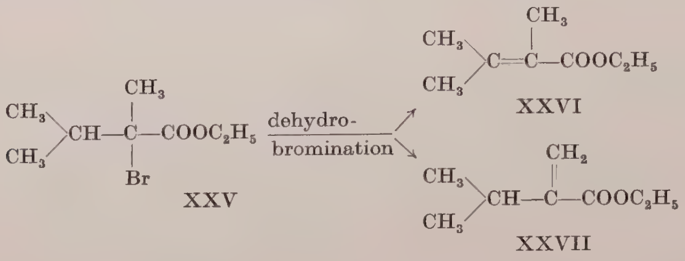
With 6 figures in the text

"Phthioic acid", the biologically active fraction of the fatty acids from the tubercle bacillus, has been assumed to be saturated (1). However, the optical rotation, $[M]_D + 50°$, is higher than that of any methyl substituted saturated acid so far synthesized, with but one exception, *viz.* the 2,3-dimethylsubstituted long chain acids derived from the enantiomorphs of 3,4-dimethyladipic acid (Part X (2)). The properties of the natural product could not be explained on the basis of the 3, 13, 19-trimethyltricosanoic acid hypothesis of POLGAR and ROBINSON (3) (see *e.g.* Part V (4)). Recently CHANLEY and POLGAR (5), and CASON and SUMRELL (6), independently found that "phthioic acid" contains α,β-unsaturated acids with a methyl group in α-position. There must also be a side chain in the γ or δ position, as the high optical rotation indicates an optically active centre in a position fairly close to the double bond. Optically active acids of this type have previously not been described, and in order to study their properties the synthesis of long chain acids of this type was undertaken. Optically active β-methyl substituted long chain acids are readily prepared from the enantiomorphs of methyl hydrogen β-methylglutarate (Parts II (7) and VI (8)), and form a suitable starting material. The route to optically active 2,5-dimethyl substituted unsaturated acids is outlined in Chart I.

Methyl 3D-methylnonadecanoate (III) was obtained in 25 % yield from L(+)-methyl hydrogen β-methylglutarate (I) and palmitic acid (II) via the KOLBE reaction (Part VI (8)). Hydrolysis of the ester gave (+)-*3D-methylnonadecanoic* acid (IV) of m.p. 47.8–48.2°, $[\alpha]_D^{23} + 4.20°$ in chloroform. The acid was converted into (+)-*3D-methylnonadecanol-1* (V) by means of lithium aluminium hydride using the procedure of NYSTROM and BROWN (9), the yield being 96 % of the theoretical. The alcohol is dimorphic with melting points at 42.8–42.9° and 31.0–31.2° respectively, and has $[\alpha]_D^{22} + 2.23°$ (chloroform).

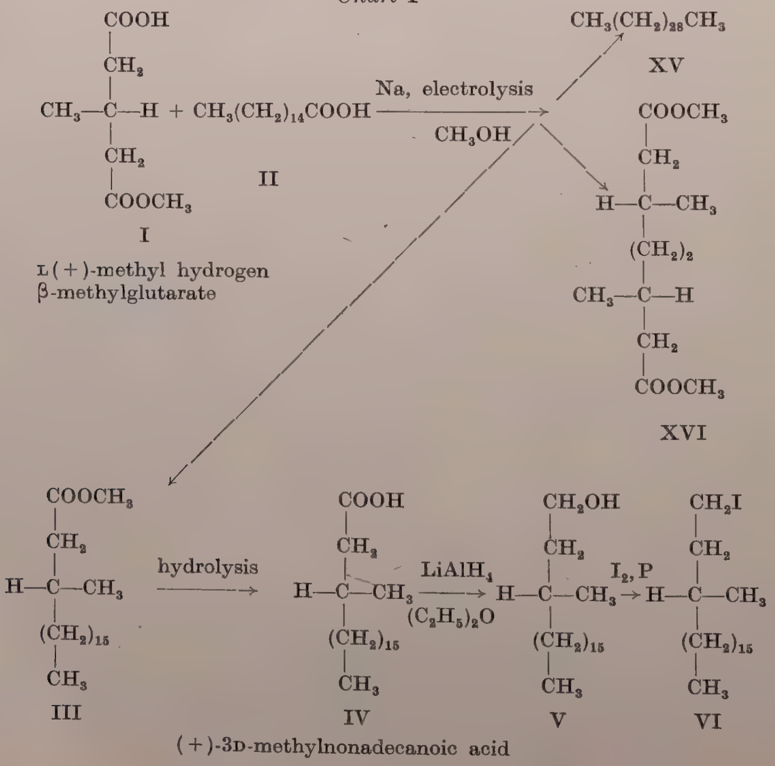
(–)-*1-Iodo-3D-methylnonadecane* (VI) was prepared from the alcohol in 93 % yield by means of iodine and red phosphorus using the usual procedure. The iodide has m.p. 21.5–22.0° and $[\alpha]_D^{20} - 6.92°$ (chloroform).

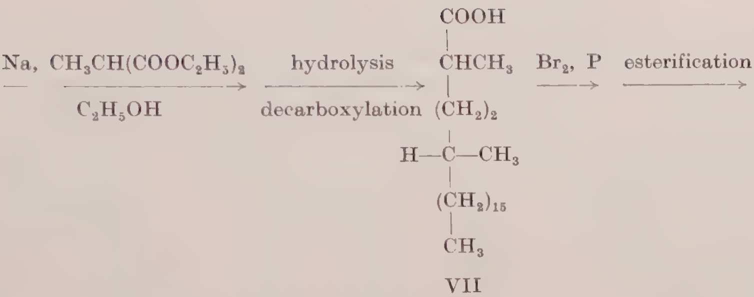
Alkylation of ethyl methylmalonate with the iodide (VI) followed by hydrolysis gave a mixture of diastereoisomeric 2,5D-dimethylheneicosanoic acids (VII). The acids (VII) were converted into a mixture of diastereoisomeric 2-bromo-2,5D-dimethylheneicosanoic acids by means of bromine and red phosphorus. The mixture of bromo-acids was converted to methyl esters, and the latter subjected to dehydrobromination by heating with quinoline or tri-*n*-propylamine.


dehydro-
bromination
XXV
XXVI
XXVII

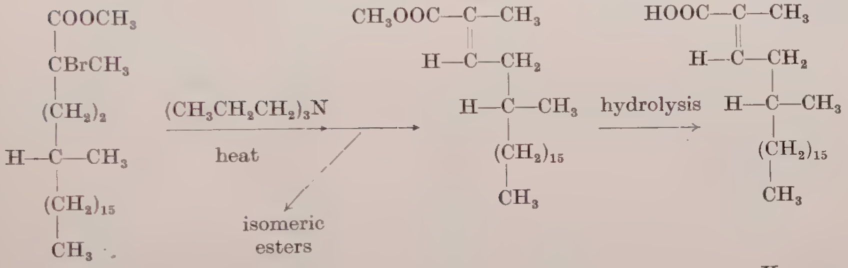
The dehydrobromination may be expected to give a mixture of isomeric unsaturated esters. RUPE, RONUS and LOTZ (10) have shown that dehydrobromination of ethyl esters of normal chain α-bromo-acids with quinoline or diethylaniline gives a mixture of α,β- and β,γ-unsaturated esters. In the present case we have a methyl group in α-position which makes further isomerism possible. W. H. PERKIN jun. found (11) that dehydrobromination of the ethyl ester of α-bromo-α,β,β-trimethylpropionic acid (XXV) gave a mixture of the α,β-unsaturated ester (XXVI) and the α-methylene ester (XXVII) with the former predominating.

Chart I


I
II
L(+)-methyl hydrogen
β-methylglutarate
Na, electrolysis
CH₃OH
XV
XVI
hydrolysis
LiAlH₄
(C₂H₅)₂O
I₂, P
III
IV
V
VI
(+)-3D-methylnonadecanoic acid


Na, $CH_3CH(COOC_2H_5)_2$
C_2H_5OH
hydrolysis
decarboxylation
COOH
$CHCH_3$
$(CH_2)_2$
$H—C—CH_3$
$(CH_2)_{15}$
CH_3
Br_2, P
esterification

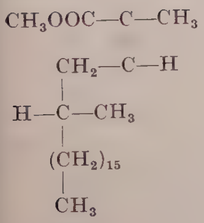
VII


$COOCH_3$
$CBrCH_3$
$(CH_2)_2$
$H—C—CH_3$
$(CH_2)_{15}$
CH_3
$(CH_3CH_2CH_2)_3N$
heat
isomeric esters
$CH_3OOC—C—CH_3$
$H—C—CH_2$
$H—C—CH_3$
$(CH_2)_{15}$
CH_3
hydrolysis
$HOOC—C—CH_3$
$H—C—CH_2$
$H—C—CH_3$
$(CH_2)_{15}$
CH_3

VIII IX X

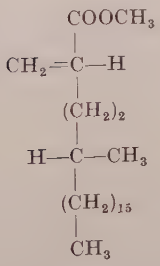
(−)-methyl *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoate *trans*-α,β

trans-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid


$CH_3OOC—C—CH_3$
$CH_2—C—H$
$H—C—CH_3$
$(CH_2)_{15}$
CH_3

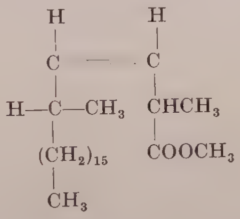
XI

cis-α,β


$COOCH_3$
$CH_2=C—H$
$(CH_2)_2$
$H—C—CH_3$
$(CH_2)_{15}$
CH_3

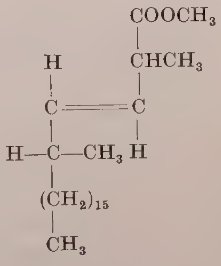
XII

α-methylene


H
H
C
C
$H—C—CH_3$
$CHCH_3$
$(CH_2)_{15}$
$COOCH_3$
CH_3

XIII

cis-β,γ


$COOCH_3$
H
$CHCH_3$
C
C
$H—C—CH_3$ H
$(CH_2)_{15}$
CH_3

XIV

trans-β,γ

Note on nomenclature:

As the α,β-unsaturated pair (IX and XI), which possess four different substituents attached to the carbon atoms forming the double bond, are considered as derived from *trans*-$\Delta^{2:3}$-heneicosenoic acid, it follows that the designation *trans* refers to the isomer (IX) that carries the carboxyl group and the alkyl chain on opposite sides of the double bond.

The formulae given in Charts I and II are written according to the extended FISCHER convention (2, 12, 13). This requires that carbon atom number 1 is placed at the top of the projection. However, it is of course sufficient for the correct representation of the optical isomerism that the numbering of the part of the molecule containing optically active centres proceeds from above downwards. The formulae of the unsaturated compounds in the charts have therefore been written in such a way as to show both the geometrical and the optical isomerism.

Table I[1]. Molecular rotations at 25°.

	Wavelength Å					
	3131	3650	4046	4358	5461	5893
(+)-Methyl 3D-methylnonadecanoate (homogeneous)[2]		+ 23.2	+18.2	+15.1	+ 8.9	+ 7.7
(+)-3D-Methylnonadecanol-1 (chloroform; *c*, 10.80) . .	+30.7	+ 20.0	+15.6	+13.2	+ 8.5	+ 6.8
(–)-1-Iodo-3D-methylnonadecane (chloroform; *c*, 10.04)		– 109.9	– 76.7	– 61.0	– 33.0	– 26.6
(–)-Methyl *trans*- 2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoate (homogeneous)[2]			– 13.8	– 10.1	– 3.85	– 2.9

[1] The data were obtained with the use of a photoelectric ultraviolet polarimeter of the Bruhat-Guinier type, built in this laboratory.

[2] At 23° (*l*, 0.5 (micro tube)).

The dehydrobromination of methyl 2DL-bromo-2DL,5D-dimethylheneicosanoate (VIII) might thus be expected to yield a mixture of several isomeric unsaturated esters. The five possible structures are shown in Chart I, (IX) and (XI–XIV).

Dehydrobromination of the methyl esters (VIII) by means of quinoline or tri-*n*-propylamine at a temperature of 155° proceeded slowly, and it was necessary to extend the treatment for periods up to 20 hours in order to get a bromine-free product.

The products of the dehydrobromination were separated by chromatography on aluminium oxide. The results of such a separation are shown in Fig. 1 and Table II. (–)-*Methyl* trans-*2,5*D-*dimethyl*-$\Delta^{2:3}$-*heneicosenoate* (IX), $[\alpha]_D^{23}$ – 0.7° ($[M]_D^{23}$ – 2.8°) in chloroform, was obtained in yields of about 15 % of the theoretical. The ester is

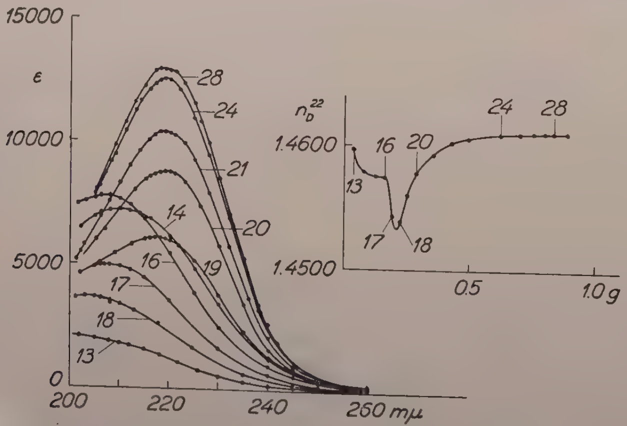


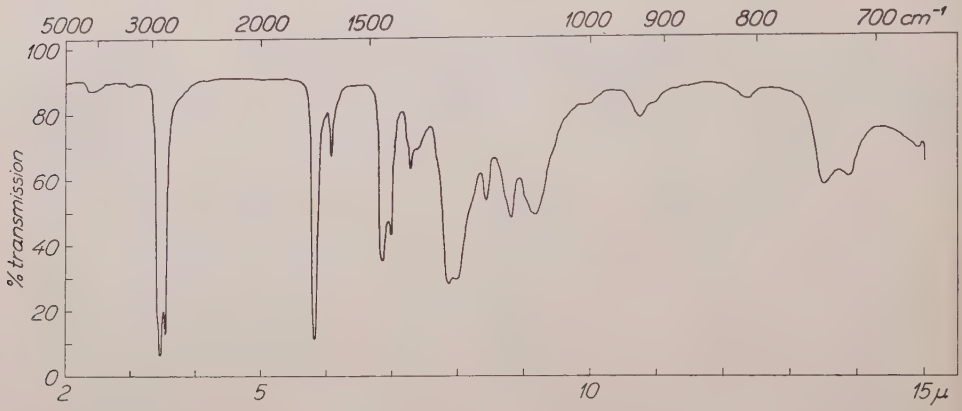
Fig. 1. Ultraviolet absorption and refractive index of fractions obtained in the chromatography of products obtained on dehydrobromination of methyl 2DL-bromo-2DL,5D-dimethylheneicosanoate (Table II).

Table II. Chromatography of products obtained on dehydrobromination of methyl 2DL-bromo-2DL,5D-dimethylheneicosanoate.

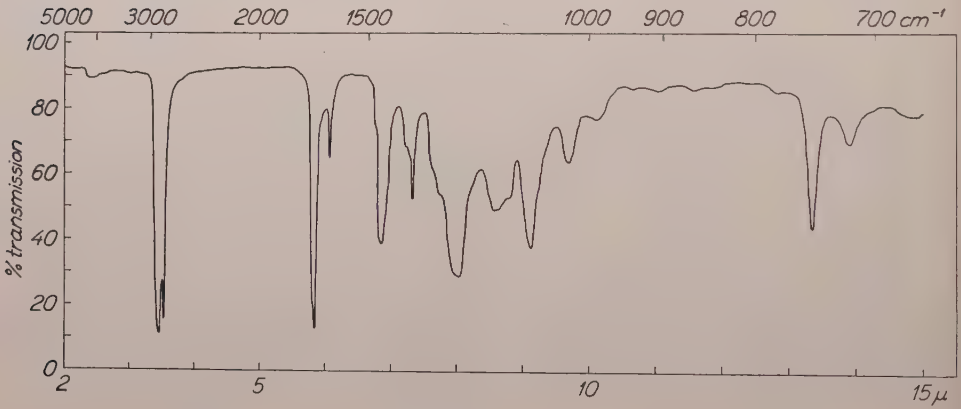
Fraction no.	Volume of effluent ml	Weight of fraction mg	Refractive index n_D^{22}	Hydrogen number	Optical rotation in light petroleum (l, 1)
1	50	1			
2	35	2			
3	26	5			
4	10	7			
5	10	6			
6	10	2			
7	10	1			
8	10	1			
9	10	2			
10	10	0			
11	10	1			
12	10	7			
13	10	35	1.4597	384	α_D^{23} −0.07° (c, 1.5)
14	10	42	1.4597		α_D^{23} −0.06° (c, 1.85)
15	12	50	1.4575	406	α_D^{23} −0.05° (c, 1.25)
16	10	32	1.4573		
17	10	30	1.4543	548	
18	10	31	1.4539	502	
19	10	31	1.4560		
20	10	32	1.4578		
21	20	76	1.4592		
22	20	70	1.4603		
23	22	66	1.4605		
24	50	125	1.4609		α_D^{23} −0.04° (c, 5.8)
25	50	79	1.4611		
26	50	59	1.4611		
27	50	41	1.4611	351	
28	50	30	1.4612		
29	50	26	1.4612		
30	50	16	1.4613		
31	50	10	1.4613		
32	50	8	–		
	Total weight 0.92 g				

dimorphic with melting points at 8.0–8.5° and 14° respectively. The ultraviolet absorption curve was found to have a maximum at 218 mμ with $\varepsilon_{\max}$ 13 000 (cf. Fig. 3). The nature of the other fractions will be discussed below. Hydrolysis of the ester (IX) gave trans-*2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid* (X), m.p. 39.6–40.0°, $[\alpha]_D^{23} \pm 0.00°$ (chloroform; l, 1; c, 20). The acid has $\varepsilon_{\max}$ 12 600 ($c = 10.2 \cdot 10^{-5}$) at 217–218 mμ, the position and height of the maximum varying slightly with concentration (Fig. 4). The infrared absorption spectrum is shown in Fig. 2c.

After the work just described had been completed there appeared a paper by Hunter and Popják (14) in which they report failure to dehydrobrominate methyl esters of normal chain α-bromo-acids by means of quinoline or diethylaniline, whereas the corresponding ethyl esters could be dehydrobrominated. The highest yields of dehydrobrominated products were, however, obtained from the neopentyl esters.


5000
3000
2000
1500
1000
900
800
700 cm⁻¹
100
80
60
40
20
0
% transmission
2
5
10
15 μ

a.


5000
3000
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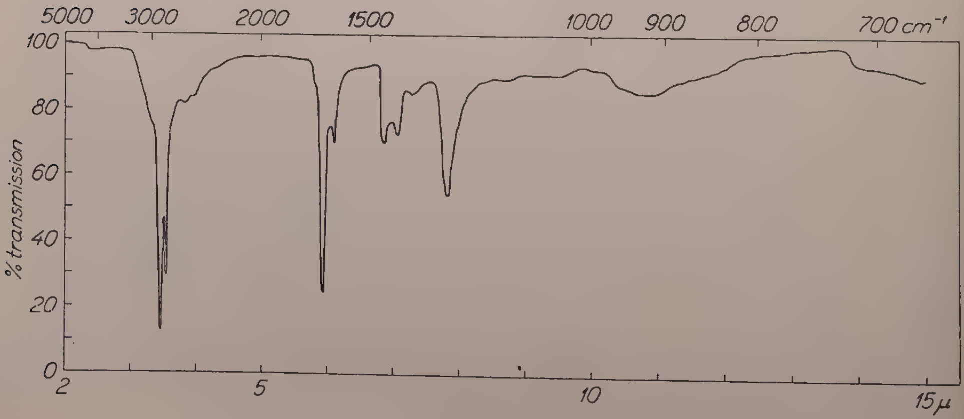
b.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% transmission
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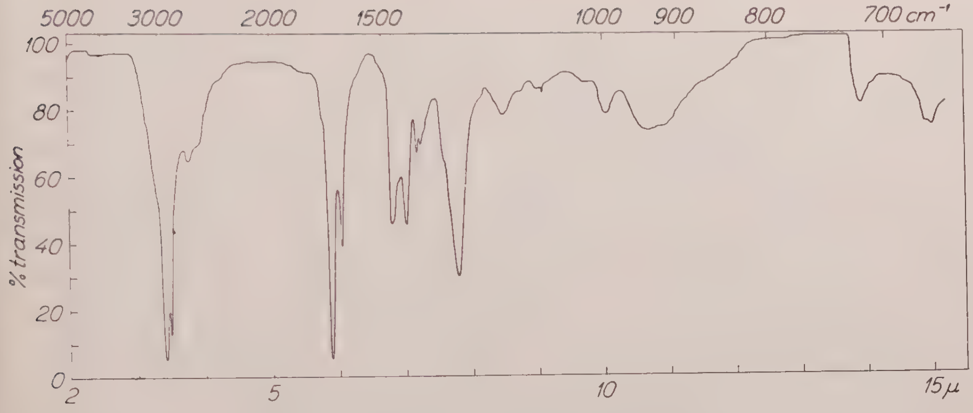
c.



d.

Fig. 2. Infrared absorption spectra.
a) (–)methyl *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenaote.
b) (–)-ethyl *trans*-2,4D-methyl-$\Delta^{2:3}$-heneicosenoate.
c) *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid. $c = 9.3 \cdot 10^{-2}$ moles per litre.
d) (–)-*trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid. $c = 2.2 \cdot 10^{-1}$ moles per litre.
The esters were run as liquids in cells 0.0125 mm thick, the acids as solutions in carbon tetrachloride in 0.1 mm cells.

HUNTER and POPJÁK used much shorter heating times than those found necessary in the work just described, and the results are therefore not strictly comparable, but it is evident that the presence of a methyl side chain in α-position makes dehydrobromination of the methyl ester possible. JOCELYN and POLGAR (15) have recently shown that dehydrobromination of the methyl esters of α-brominated acids by means of pyridine is possible when there is a methyl side chain in α- or β-position. Dehydrobromination of the α-bromo methyl ester derived from (–)-4,8-dimethylnonanoic acid failed.

The route to optically active 2,4-dimethyl-substituted α,β-unsaturated acids is shown in Chart II. (+)-*3D-Methyleicosanoic acid* (XVI), m.p. 52.2–52.4°; $[\alpha]_D^{23}$ + +4.00° ($[M]_D^{23}$ + 13.1°) in chloroform, was prepared by hydrolysis of the methyl ester obtained in 27 % yield from (I) and *n*-heptadecanoic acid in a KOLBE synthesis analogous to that used in the case of the lower homologue (III). (+)-*Methyl 3D-methyleicosanoate* is dimorphic, the melting points being 30.1–30.4° and 21.1–21.3° respectively.

The anodic synthesis involving a mixture of (+)-3D-methyleicosanoic acid (XVI) and ethyl hydrogen methylmalonate (XVII) gave a mixture of the ethyl esters of the stereoisomeric 2D,4D- and 2L,4D-dimethylheneicosanoic acids (XX), together with the C_{40} hydrocarbon (XVIII) and the diester (XIX). Other by-products of the reaction are 2-methylnonadecane and 2-methylnonadecene-1, produced by disproportionation of the radical formed after decarboxylation of the discharged carboxylate ion. It is stated in the literature that the presence of an α-alkyl substituent in most cases lowers the yields of the coupled product in the KOLBE reaction (16). However, by using a molecular ratio of 4.5 : 1 between ethyl hydrogen methylmalonate and (+)-3D-methyleicosanoic acid, a yield of 25% of the theoretical of the coupled product was obtained, calculated on the used-up amount of the optically active acid (XVI).

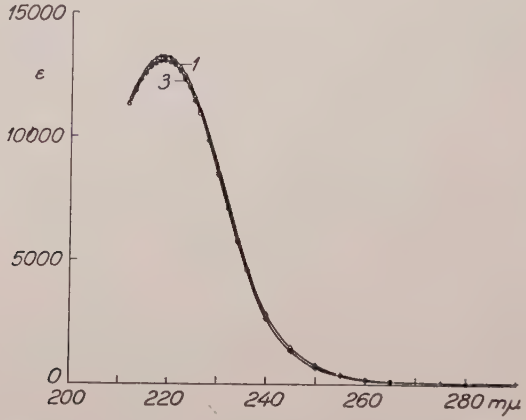


Fig. 3. Ultraviolet absorption of methyl *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoate. Curve 1 ($\Delta - \Delta$) c = $2.14 \cdot 10^{-5}$; 2: (+ – +) c = $8.5 \cdot 10^{-5}$; 3: (0–0) c = $4.3 \cdot 10^{-5}$ moles per litre.

In later experiments of the same type it was found possible to increase the yield of the desired product by using a still larger excess of ethyl hydrogen methylmalonate. Most of the half-ester does not couple, and the radical formed after the carboxylate ion has been discharged and decarboxylated is evidently disproportionated into a mixture of acrylic and propionic esters.

The products of the reaction were separated by means of distillation and chromatography on aluminium oxide.

The ethyl esters (XX) were hydrolysed, and the acids (XXI) α-brominated by the usual procedure. For reasons already discussed the α-bromo-acid bromides were converted into the *ethyl* esters (XXII), and the latter subjected to dehydrobromination.

Separation of the products by means of chromatography on aluminium oxide gave (–)-*ethyl* trans-*2,4*D*-dimethyl-*$\Delta^{2:3}$*-heneicosenoate* (XXIII) of m.p. 23.6–24.0° and

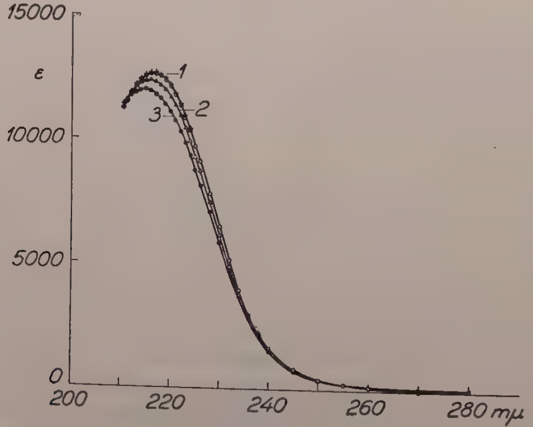
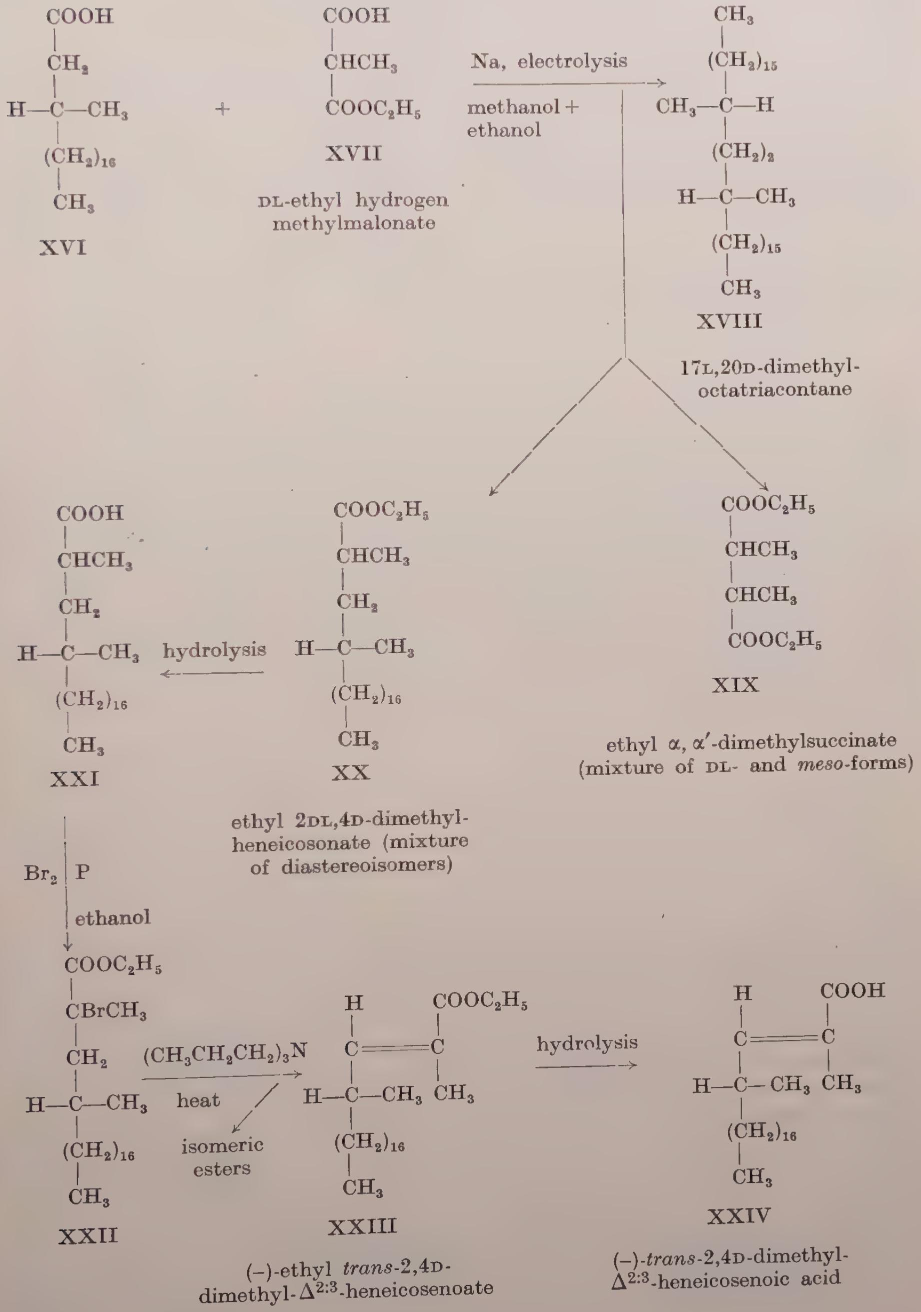


Fig. 4. Ultraviolet absorption of *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid. Curve 1: c = $10.2 \cdot 10^{-5}$, 2: c = $5.1 \cdot 10^{-5}$; 3: c = $2.55 \cdot 10^{-5}$ moles per litre.

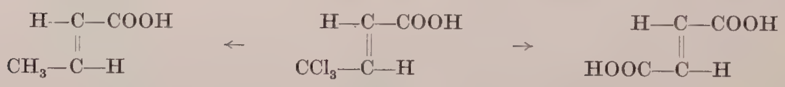
Chart II


XVI
+
XVII
DL-ethyl hydrogen methylmalonate
Na, electrolysis
methanol + ethanol
XVIII
17L,20D-dimethyl-octatriacontane
XIX
ethyl α, α′-dimethylsuccinate (mixture of DL- and *meso*-forms)
XX
ethyl 2DL,4D-dimethyl-heneicosonate (mixture of diastereoisomers)
hydrolysis
XXI
Br_2 P
ethanol
XXII
$(CH_3CH_2CH_2)_3N$
heat
isomeric esters
XXIII
(−)-ethyl *trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoate
hydrolysis
XXIV
(−)-*trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid

$[M]_D^{22}$ − 84° in light petroleum. A low-melting form of the ester melting at 7° has also been observed. The ultraviolet absorption curves of Fig. 5 show ε_{max} 13100 at 218 mμ. The infrared absorption curve is reproduced in Fig. 2 b.

Hydrolysis of the ester gave (−)-trans-*2,4*D*-dimethyl-*$\Delta^{2:3}$*-heneicosenoic acid* (XXIV). The acid is dimorphous, the observed melting points being 46.0–46.2° and 49.6–50.2° respectively. The specific rotation in chloroform was found to be $[\alpha]_D^{23}$ − 21.9° corresponding to a molecular rotation $[M]_D^{23}$ of − 77°.

The assignment of the *trans*-configuration to the α,β-unsaturated compounds described above is based on analogy and not on direct proof. In the first place, dehydrobromination of ethyl α-bromobutanoate by means of diethylaniline gives



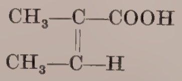
$$\begin{array}{c}H-C-COOH\\ \|\\ CH_3-C-H\end{array} \leftarrow \begin{array}{c}H-C-COOH\\ \|\\ CCl_3-C-H\end{array} \rightarrow \begin{array}{c}H-C-COOH\\ \|\\ HOOC-C-H\end{array}$$

XXVIII XXIX XXX

crotonic acid γ,γ,γ-trichlorocrotonic acid fumaric acid

the ethyl ester of crotonic acid (17). The configuration of crotonic acid as *trans*-$\Delta^{2:3}$-butenoic acid has been established by chemical methods by v. AUWERS and WISSEBACH (18), who showed that oxidation and reduction respectively of γ,γ,γ-trichlorocrotonic acid (XXIX) under conditions very unlikely to cause steric rearrangement gives fumaric acid (XXX) and crotonic acid (XXVIII).

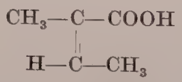
Further evidence for the *trans*-structure of the α,β-unsaturated long chain acids is obtained from a comparison of their ultraviolet absorption with that of tiglic acid (*trans*-2-methyl-$\Delta^{2:3}$-butenoic acid (XXXI)) and angelic acid (*cis*-2-methyl-$\Delta^{2:3}$-butenoic acid (XXXII)).



$$\begin{array}{c}CH_3-C-COOH\\ \|\\ CH_3-C-H\end{array}$$

XXXI

tiglic acid



$$\begin{array}{c}CH_3-C-COOH\\ \|\\ H-C-CH_3\end{array}$$

XXXII

angelic acid

The ultraviolet absorption of these acids has been determined in the course of the present work. The acids were prepared as described by BUCKLES and MOCK (19). For tiglic acid ε_{max} was found to be 11500 at 213 mμ ($c = 5 \times 10^{-5}$ moles per litre), and for angelic acid $\varepsilon_{max} = 8400$ at 215 mμ ($c = 5 \times 10^{-5}$). As the configurations of tiglic and angelic acids may be considered as rather firmly established (20), the figures just given favour the *trans*-configuration of the long chain acids.

A definite proof of the configuration of geometrical isomers of this type can of course be obtained by detailed X-ray crystallographic investigations. So far, structure proof from such studies appears to be available only in the case of fumaric acid. KNAGGS and LONSDALE (21) found that dimethyl fumarate is triclinic with space group $P\bar{1}$ (C_i^1). As there is one molecule in the cell and the space group has a centre of symmetry it follows that the molecule itself must have a centre of symmetry. This is the case only for the *trans*-configuration.

As described in detail in the experimental part, the *trans*-α,β ester fractions are in the chromatographic separation preceded by several other fractions. These fractions have not been studied in detail in the course of the present work.[1] The saturated fraction of low refractive index observed in front of the *trans*-2,5D-methyl ester (see Fig. 1) may consist of unchanged starting material or of the lactide ester of the α-hydroxy acid (cf. Hunter and Popják (14)). Both in the case of the 2,4D- and 2,5D-compounds there is obtained a fraction whose absorption maximum in the ultraviolet lies at 208-210 mμ with $\varepsilon_{max} \sim 7500$. The infrared spectrum of these fractions shows a slight displacement of the ethylene band towards the long-wave side. In the case of the *trans*-2,5D-ester (IX) (fraction 28 of Fig. 1) this band occurs at 6.07 μ, whereas fraction 14 of Fig. 1 has the corresponding band at 6.14 μ. This fraction has an optical rotation $[M]_D \sim + 30°$; the sign is thus opposite to that of the *trans*-α,β-unsaturated ester. A comparison of the above data with those for long chain α-methylene substituted acids recently prepared by Professor James Cason and his collaborators at Berkeley indicates a α-methylene structure of the chromatographic fraction in question.[2]

When the bromination is carried out with bromine and red phosphorus there appears to be no danger of racemization due to attack of bromine at tertiary carbon atoms not in α-position to carboxyl. Catalytic hydrogenation of the unsaturated product obtained by dehydrobromination of 2DL-bromo-19D-methylheneicosanoic acid gave (–)-19D-methylheneicosanoic acid with melting point and optical rotation identical with that of the acid used as starting material.[3]

The amount of β,γ-unsaturated material found in the dehydrobrominations, which in the case of the 2,4D-compounds would be optically inactive, appears to be very small. Similar dehydrobrominations of normal chain α-bromo esters give considerable amounts of β,γ-unsaturated product (10). A low mobility of the α,β⇌β,γ-change is to be expected, as the extensive work of Linstead and Kon and their collaborators in this field (22, 23) shows that the presence of side chains may considerably reduce the tautomeric mobility. Apparently no 2,4-dimethylsubstituted acids have been investigated, but *e.g.* esters of 2,3-dialkylsubstituted acids show a high degree of stability (23).

The results of the present investigations are in general agreement with those of Jocelyn and Polgar (15), who recently described the synthesis of α,β-unsaturated (–)-2,4,8-trimethylnonenoic and (–)-2,5,9-trimethyldecenoic acids starting from dihydrocitronellol, and those of Cason *et al.* on optically active 2,4-dimethyl substituted $\Delta^{2:3}$-tricosenoic acids.[2]

According to a preliminary report of Polgar and Robinson (24) the α,β-unsaturated mycolipenic acid-I (probably identical with the phthienoic acid of Cason and Sumrell (6, 25, 26)) on oxidation gave a saturated acid $C_{24}H_{48}O_2$ which after α-bromination and dehydrobromination gave an α,β-unsaturated acid the methyl ester of which had $[\alpha]_D^{21} + 13.3°$. Oxidation of this acid gave an α-methyl-substituted acid $C_{21}H_{42}O_2$ with $[\alpha]_D + 7°$. They suggest on the basis of these results that mycolipenic acid-I is 2,4,6-trimethyl-$\Delta^{2:3}$-tetracosenoic acid. Assuming the correctness of these results, the steric configuration of mycolipenic acid-I can be derived as follows.

The dextrorotation of the α-methyl substituted acid shows that it has the 2L-configuration (XXXIII) (Part I (27)). The unsaturated C_{24} acid from which it is derived is dextrorotatory, and it follows from the work described in this communication that this acid has the configuration 2,4L- (XXXIV). Hence, the dextrorotatory mycolipenic acid-I must have the structure of *trans*-2,4L,6L-trimethyl-$\Delta^{2:3}$-tetracosenoic acid (XXXV), as it seems very unlikely that the centre in position 6 will give any large contribution to the optical rotation of the molecule.

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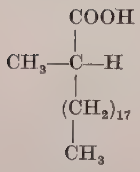
[1] It was originally intended to study the other fractions in detail in connexion with a larger scale preparation of (+)-*trans*-2,4L-dimethyl-$\Delta^{2:3}$-heneicosenoic acid (antipode of the 2,4D-acid described), but owing to unforeseen circumstances this work has had to be postponed.

[2] Personal communication from Professor Cason. The author wishes to express her thanks to him for kindly giving details prior to publication of work carried out at Berkeley.

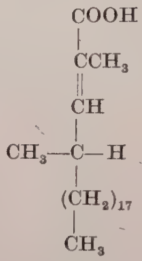
[3] Unpublished.



$HOOC-CH(CH_3)-(CH_2)_{17}-CH_3$

2L-

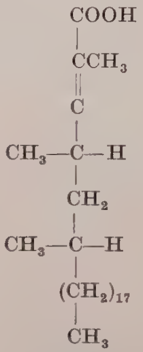
XXXIII



$HOOC-C(CH_3)=CH-CH(CH_3)-(CH_2)_{17}-CH_3$

2,4L-

XXXIV



$HOOC-C(CH_3)=C-CH(CH_3)-CH_2-CH(CH_3)-(CH_2)_{17}-CH_3$

2,4L,6L-

XXXV

Experimental[1, 2]

(+)-Methyl 3D-methylnonadecanoate (III)

The ester was prepared in 25% yield by mixed electrolysis of (+)-methyl hydrogen β-methyl glutarate (28) (35 g) and palmitic acid (56 g), following the procedure described in detail previously (29). The palmitic acid used had been carefully purified via the methyl ester and had a melting point of 62.6–62.9°. FRANCIS and PIPER (30) give m.p. 62.9°.

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[1] Melting points, long crystal spacings and optical rotations were determined as described in Part I of this series (27), cf. also (13).

[2] Spectroscopic measurements. Ultraviolet absorption measurements were carried out in 95 % ethanol solution in 1 cm cells using a BECKMAN Model DU quartz spectrophotometer. The solvent was purified as described by HARRISON *et al.* (31). Only fresh solutions that had not been exposed to strong daylight were used. The instrument was checked by measuring the extinction values for potassium chromate in 0.05 N potassium hydroxide. The agreement with the values given by CANNA, COLLINS and MORTON (32) was excellent. The stray light in the region below 220 mμ was determined as described by SAIDEL, GOLDFARB and KALT (33). It was found that in the particular instrument used stray light corrections had to be applied at 205 mμ and below. The corrections were calculated with the aid of the experimentally found data given below (cf. SAIDEL *et al.*). The author is indebted to Dr. H. LJUNGBERG, Ulleråker Hospital, for the loan of this instrument.

200 mμ	A = 1.24	α = 0.057
202	2.1	0.008
205	2.7	0.002

The infrared absorption curves were recorded on a PERKIN-ELMER Model 21 double-beam instrument in the Institute of Biochemistry. The author is indebted to Professor A. TISELIUS for permission to use this instrument. Experimental details are given in the text to Fig. 2.

The reaction mixture was worked up as described (29) and fractional distillation of the products from the electrolysis gave 17.9 g of colourless (+)-*methyl 3*D-*methylnonadecanoate*, b.p. 147–149°, 0.2 mm. A specimen of the methyl ester prepared by means of diazomethane from the acid described below, melted, after crystallization from light petroleum (b.p. 20–40°), at 25.6–25.8°. On cooling, solidification of the melt occurred at 15.0°, and the ester remelted at 15.2–15.4°. After melting, the higher melting form began to crystallize out. d_4^{23} 0.859.

Long crystal spacing (high-melting form) 29.1 Å

Optical rotation:

α_D^{23} + 0.96° (homogeneous; *l*, 0.5)
$[\alpha]_D^{23}$ + 2.24° $[M]_D^{23}$ + 7.30°
α_D^{23} + 0.30° ± 0.02° (chloroform; *l*, 1; *c*, 7.88)
$[\alpha]_D^{23}$ + 3.8° $[M]_D^{23}$ + 12.4° ± 1.0°

Anal.:	Calcd for $C_{21}H_{42}O_2$ (326.5)	C, 77.24; H, 12.97
	Found	C, 77.44; H. 12.92

(+)-3D-Methylnonadecanoic acid (IV)

The methyl ester (25 g) was hydrolyzed by refluxing for four hours with potassium hydroxide (15 g), water (10 ml), and ethanol (150 ml). Crystallization from light petroleum (b.p. 40–60°) at a temperature of −15° gave a slightly yellow acid (23.2 g) of m.p. 47.5–48.0°. After recrystallization from acetone at −15° (+)-*3*D-*methylnonadecanoic acid* formed colourless lustrous plates melting at 47.8–48.2°. Yield 22.1 g.

Long crystal spacing (pressed specimen) 33.2 Å

Optical rotation:

α_D^{23} + 1.79° (chloroform; *l*, 4; *c*, 10.65)
$[\alpha]_D^{23}$ + 4.20° $[M]_D^{23}$ + 13.1°

Anal.:	Calcd for $C_{20}H_{40}O_2$ (312.5)	C, 76.86; H, 12.90 %
	Found	C, 76.90; H, 12.89.%

(+)-3D-Methylnonadecanol-1 (V)

Lithium aluminium hydride (7 g) was pulverized and dissolved in dry ether (150 ml). The practically clear ether solution was pipetted off from undissolved material and transferred to a 500 ml round-bottomed flask. A solution of (+)-3D-methylnonadecanoic acid (16.8 g) in dry ether (150 ml) was added dropwise to the $LiAlH_4$ solution. When all had been added the solution was refluxed for ½ hour. Water was added, followed by dilute sulphuric acid. The mixture was transferred to a separating funnel, the ether layer separated and dried with sodium sulphate, and the solvent distilled off. The white residue crystallized from light petroleum (b.p. 40–60°) at −15°, giving 15.5 g (96% of the theoretical) of (+)-*3*D-*methylnonadecanol-1*, m.p. 42.8–42.9°. The alcohol solidified at 30.8° and remelted at 31.0–31.2°. The low-melting form is produced by slow cooling of the melt, whereas the high-melting form is obtained on rapid cooling or on keeping the melt for some time at a temperature just above the m.p. of the low-melting form.

Long crystal spacing (high-melting form) 28.0 Å

Optical rotation:

$\alpha_D^{22} + 0.95°$ (chloroform; *l*, 4; *c*, 10.63)
$[\alpha]_D^{22} + 2.23°$ $[M]_D^{22} + 6.67°$

Anal.:	Calcd for $C_{20}H_{42}O$ (298.5)	C, 80.46; H, 14.18 %
	Found	C, 80.24; H, 14.16 %

(–)-1-Iodo-3D-methylnonadecane (VI)

The alcohol just described (14.4 g) was converted into the iodide by means of iodine (6.73 g; 10% excess) and red phosphorus (0.5 g). The mixture was kept at a temperature of 145° for 5 hours in an oil bath. The reaction mixture was extracted with ether and the ether solution washed with water, 5% sodium hydroxide solution, and water. The ether solution was dried with sodium sulphate, the solvent distilled off, and the residue dissolved in acetone. When the solution was kept at a temperature of – 18° *(–)-1-iodo-3D-methylnonadecane* (19.7 g or 93% of the theoretical) crystallized in the form of colourless lustrous plates. M.p. 21.5–22.0°.

Optical rotation:

$\alpha_D^{20} - 2.89°$ (chloroform; *l*, 4; *c*, 10.44)
$[\alpha]_D^{20} - 6.92°$ $[M]_D^{20} - 28.2°$

Anal.:	Calcd for $C_{20}H_{41}J$ (408.5)	J, 31.07 %
	Found	J, 31.18 %

2DL,5D-Dimethylheneicosanoic acid (VII) (mixture of diastereoisomers)

Sodium (2 g, 0.087 atom) was dissolved in dry ethanol, ethyl methylmalonate (22.6 g, 0.13 mole) added, followed by (—)-1-iodo-3D-methylnonadecane (17.7 g, 0.0433 mole). The mixture was refluxed (calcium chloride tube) for 7 hours.

The reaction mixture was acidified and extracted with ether. The ether solution was washed with water and dried with sodium sulphate. The solvents were distilled off. The residue was added to a solution of 10% potassium hydroxide in ethanol and the mixture refluxed for 5 hours. The dicarboxylic acid was isolated in the usual manner and without further purification decarboxylated by being slowly heated to a temperature of 200° in a round-bottomed flask. After the evolution of the gas had subsided the flask was cooled to room temperature. After crystallization from light petroleum 15 g (86% of the theoretical) of a mixture of the 2D,5D- and 2L,5D-dimethylheneicosanoic acids melting at 28.5–30.5° was obtained.

In order to compare the properties of the corresponding diastereoisomeric methyl esters with fractions obtained in the chromatographic separations, 0.72 g of the acids were esterified by means of diazomethane. The mixture of esters were dissolved in light petroleum (b.p. 40–60°) and the solution passed through a column of 50 ml Al_2O_3. A total of 0.73 g of methyl esters, m.p. 9.3–9.6° was obtained. $n_D^{21.3}$ 1.4472.

$d_4^{21.3}$ 0.8587 $\alpha_D^{22} - 0.44°$ (homogeneous; *l*, 0.5).

(−)-Methyl *trans*-2,5D-Dimethyl-$\Delta^{2:3}$-heneicosenoate (IX)

Bromination of 2DL,5D-dimethylheneicosanoic acid (12 g, 0.0338 mole) was carried out by the general procedure described by MENDEL and COOPS (34). Red phosphorus (1.05 g) was added to the acid, the mixture heated to 80° and bromine (21.7 g) added dropwise. The reaction mixture was kept at 80° for 5 hours. As a result of an accident the acid bromide was contaminated with water, and the reaction mixture was therefore poured into water. After stirring for 15 minutes the mixture was left at room temperature for 2 hours. The bromo-acid was taken up in ether, the ether solution washed with water and dried by means of sodium sulphate. Diazomethane in ether solution was added, and excess diazomethane and the solvent distilled off. The resulting crude bromo-ester of d_4^{22} 0.949 and n_D^{22} 1.4658 was subjected to dehydrobromination.

Preliminary trials were carried out with quinoline and tri-*n*-propylamine as dehydrobromination agents. Considerable darkening of the reaction mixture occurred when the former reagent was employed. Careful purification of the quinoline via the acid sulphate, and distillation before use, caused no improvement: tri-*n*-propylamine may therefore be preferable.

It was found necessary to heat the bromo-ester with the dehydrobrominating agent for periods up to 20 hours, using a metal bath kept at a temperature of 180°, in order to obtain a bromine-free product.

After cooling to room temperature ether was added to the reaction mixture and the ether solution washed several times with dilute hydrochloric acid and water. After drying over sodium sulphate the ether was distilled off. The material thus obtained was chromatographed on aluminium oxide. The columns were prepared in the manner described by REICHSTEIN and SHOPPEE (35), and light petroleum (b.p. 40–60°) was used as solvent and eluant. The details of a typical experiment are given below.

3.0 g of bromo-ester were refluxed with 2.5 ml of tri-*n*-propylamine for 20 hours. Three fifths of the dehydrobrominated product was dissolved in light petroleum, and the solution allowed to pass through a column of 150 ml of aluminium oxide (MERCK, "standardisiert nach BROCKMANN") using a tube of 25 mm internal diameter. The experimental data are collected in Table II. In Fig. 1 (inset) the refractive indices of the different fractions obtained in the chromatography are plotted as a function of the amount of methyl esters passed through the column.

The ultraviolet absorption curves for several of the fractions are given in Fig. 1. The hydrogen number, high refractive index, and high ultraviolet absorption ($\varepsilon_{\max}$ 13 000 at 218 mμ), of fractions 24–31 (0.386 g) indicate that these consist of the trans-α,β-*unsaturated ester*. Apart from the *trans*-ester, which is the largest single component, the data of Table II indicate the presence of several other components. The minute amount of solid material obtained in fractions 3–5 is cleanly separated from the rest, and on account of the small amount it was not further investigated. The hydrogen number of fraction 13 shows that it is unsaturated. It has, however, a low ultraviolet absorption, and it is possible that this fraction consists mainly of β,γ-unsaturated ester. The fractions 15–16 forming a small plateau in the refractive index curve have a higher absorption in the ultraviolet, fraction 16 having $\varepsilon_{\max}$ 7 800 at about 208 mμ. As mentioned in the introduction, it is possible that this fraction consists mainly of the α-methylene ester. The fractions 17–18 corresponding to the minimum in the refractive index curve have high hydrogen numbers and low ultraviolet absorption, and thus seem to contain a saturated component. The optical rotations recorded in Table I are small, and there are no large differences between the rotations of the fractions measured.

Similar results were obtained in other chromatographic runs using aluminium oxide. By the use of a larger amount of Al_2O_3 with respect to the amount of dehydrobrominated material somewhat better separations of the fractions were obtained; for instance, the refractive index minimum corresponding to the fraction containing saturated material could be brought to n_D^{25} 1.4500.

As is evident from Table II the amount of material per unit volume of effluent is practically constant, which indicates that the chromatogram is of the displacement type (36).

The *trans*-ester has two crystalline forms with melting points at 14° and 8.0–8.5° respectively. The infrared spectrum is reproduced in Fig. 2 a, and the ultraviolet absorption at three different concentrations is shown in Fig. 3. d_4^{23} 0.878. n_D^{23} 1.4608.

Optical rotation:

$\alpha_D^{23} - 0.07°$ (chloroform; *l*, 1; *c*, 9.3)
$[\alpha]_D^{22} - 0.7°$ $[M]_D^{22} - 2.8°$

Anal.: Calcd for $C_{24}H_{46}O_2$ (366.6) C, 78.62; H, 12.65 %
Found C, 78.30; H, 12.55 %
Hydrogen number calcd 366.6; found 369.

trans-2,5D-Dimethyl-$\Delta^{2:3}$-heneicosenoic acid (X)

trans-Methyl ester (0.93 g) was added to a solution of potassium hydroxide (1 g), water (2 ml), and ethanol (30 ml of 99.5%), and the resulting solution left overnight at room temperature (21° C). The hydrolysis mixture was worked up and the acid crystallized from 25 ml of light petroleum (b.p. 40–60°) at a temperature of −22°, giving 0.54 g of white crystalline *acid* of m.p. 39.6–40.0°. Recrystallization brought no change in the melting point. The acid crystallizes from light petroleum in the form of thin lustrous plates. Polymorphism has not been observed.

The ultraviolet absorption curves for the *trans*-acid at three different concentrations are given in Fig. 4, and the infrared absorption curve over the sodium chloride region is reproduced in Fig. 2 c.

Long X-ray spacing (pressed or melted specimen) 42.5 Å.

Optical rotation:

A 20 % solution in chloroform of the acid showed no measurable rotation in a 1 dm tube for sodium light.

Anal.: Calcd for $C_{23}H_{44}O_2$ (352.6) C, 78.34; H, 12.58 % C-methyl (Kuhn-Roth) calcd 3;
Found C, 78.70; H, 12.60 % found 2.4.

Ethyl hydrogen methylmalonate (XVII)

A solution of potassium hydroxide (9.3 g) in ethanol (250 ml of 99.5%) was added to ethyl methylmalonate (25.3 g) (37) and the solution refluxed for half an hour. Some dipotassium salt was filtered off while the mixture was still hot. Most of the ethanol was distilled off, and the residual solution cooled under running water until crystallization began. The separation of the acid potassium salt was completed by addition of ether and shaking. The crystalline salt was filtered off and dried *in vacuo* over sulphuric acid. Yield 80% of the theoretical. The salt was decomposed by dilute hydrochloric acid, and the ethyl hydrogen methylmalonate distilled *in vacuo*, b.p. 83.5°, 0.2 mm. Yield 18.2 g.

(+)-Methyl 3D-methyleicosanoate

The ester was prepared in the manner described for the lower homologue, using (+)-methyl hydrogen β-methylglutarate (20.5 g; $\alpha_D^{21.6}$ + 1.27° (homogeneous; *l*, 2), *n*-heptadecanoic acid (34.6 g), and sodium (0.5 g). The *n*-heptadecanoic acid had been carefully purified via the ethyl ester, and melted at 61.1–61.3°. FRANCIS and PIPER (30) give m.p. 61.3°. A mixture of methanol and ethanol (1:1 by volume) was used as solvent in the electrolysis. The material to be electrolyzed was added in three portions, and the electrolysis was run at 4 amps. for a total time of four and a half hours. The warm reaction mixture was poured into a large volume of ether, and the solution left at room temperature for 2 hours. The precipitate was filtered off and washed with ether. The combined ether solutions were washed with water, 5% sodium carbonate solution, and again with water. A small amount of precipitate was filtered off, and the solution dried with sodium sulphate. The ether was distilled off and the residue distilled *in vacuo*.

I	79–84°, 0.1 mm	8.3 g	α_D^{22} + 5.80°	(hom., *l*, 1)
II	85–149°, 0.1 »	2.4 »	α_D^{22} + 3.84°	(»)
III	149–153°, 0.1 »	4.75 »	α_D^{22} + 1.69°	(»)
IV	153–152°, 0.1 »	6.85 »	α_D^{22} + 1.75°	(»)

Yield of the desired *ester* (fractions III and IV) 11.6 g, or 26.6% of the theoretical.

A specimen of the ester prepared by means of diazomethane from the acid described below was crystallized from light petroleum (b.p. 40–60°). The ester was obtained in the form of large, lustrous plates of m.p. 30.1–30.4°. On cooling solidification took place at 21.0°. The crystal form now obtained melted at 21.1–21.3°.

Long crystal spacing (crystallized from light petroleum) 30.3 Å.

Optical rotation:

α_D^{22} + 0.24° ± 0.02° (chloroform; *l*, 1; *c*, 6.57)

$[\alpha]_D^{22}$ + 3.60° $[M]_D^{22}$ + 12.4° ± 1.0°

Anal.: Calcd for $C_{22}H_{44}O_2$ (340.6) C, 77.58; H, 13.02 %

Found C, 77.64; H, 13.06 %

(+)-3D-Methyleicosanoic acid (XVI)

The methyl ester (11.2 g) was refluxed with potassium hydroxide (7 g), water (14 ml), and ethanol (100 ml), for 4 hours. The hydrolysis mixture was worked up in the usual manner and the residue crystallized from 150 ml of acetone at a temperature of 0°. Yield 9.14 g of (+)-*3D-methyleicosanoic acid* in the form of lustrous plates melting at 51.8–52.5°. After recrystallization from acetone the m.p. was 52.2–52.4°.

Long crystal spacing (pressed specimen) 28.3 Å. A specimen prepared by the hot-wire technique showed an additional spacing of 35 Å.

Optical rotation:
α_D^{23} + 1.57° (chloroform; *l*, 4; *c*, 9.81)
$[\alpha]_D^{23}$ + 4.00° $[M]_D^{23}$ + 13.1°

Anal.: Calcd for $C_{21}H_{42}O_2$ (326.6) C, 77.23; H, 12.96 %
Found C, 77.28; H, 12.91 %

Ethyl 2DL,4D-dimethylheneicosanoate (XX)

(mixture of diastereoisomers)

(+)-3D-Methyleicosanoic acid (6.05 g, 0.0185 mole) and ethyl hydrogen methylmalonate (8.1 g, 3×0.0185 mole) where subjected to electrolysis in a solution of sodium (0.35 g) in methanol (300 ml). The acids were added in two portions. When the reaction was neutral after the second addition, 4 g of methyl hydrogen methylmalonate was added and the electrolysis continued until the reaction mixture was weakly alkaline. A volatile ester-smelling product with lachrymatory properties (probably a mixture of propionic and acrylic esters) was produced. Water and ether were added (together with a small amount of hydrochloric acid to prevent foam formation). The ether solution was washed twice with water, followed by 5% sodium carbonate solution. A rather persistent emulsion was formed. The mixture was therefore again acidified with dilute hydrochloric acid and no further attempts were made to remove acidic products at this stage. The ether was washed with water. After drying with sodium sulphate the solvent was distilled off and the residue distilled *in vacuo*.

I	48°	0.2 mm	5.09 g
II	48 –170°	0.2 »	0.5 ml
III	170–174°	0.2 »	2.94 g
IV	174°	0.2 »	1.04 g

Fraction I consists of a mixture of stereoisomeric α,α′-dimethyl succinates (XIX). Fraction III and IV contain the desired ester (XX).

The residue in the flask was solid at room temperature. This residue contains 17L,20D-dimethyloctatriacontane (XVIII). This hydrocarbon was not further studied in the course of the present work.

1.48 g of fraction III was chromatographed on 75 ml of aluminium oxide, yielding 0.45 g of material melting at 10.4–11.4°. A second run, using 100 ml of Al_2O_3 and the rest of fraction III + fraction IV, gave a further 0.83 g of material with the same m. p.

The aluminium oxide used was treated with dilute hydrochloric acid. Extraction with ether and crystallization of the organic material obtained from acetone gave 1.65 g of (+)-3D-methyleicosanoic acid of m.p. 51.7–52.3°.

Chromatography of fraction II on 70 ml of Al_2O_3 gave 0.46 g of neutral, optically inactive material of m.p. 17.3°–17.8°, evidently consisting of 2-methylnonadecane. This was followed by a smaller neutral fraction of m.p. 12–13°, probably consisting mainly of 2-methylnonadecene-1. The melting points of these two hydrocarbons are reported as 18.3° and 11–12° respectively (38). This was followed by fractions containing small amounts of (XX). Finally, there emerged strongly-smelling fractions containing ethyl α,α′-dimethylsuccinates (XIX).

Yield of (XX), corrected for recovered (+)-3D-methyleicosanoic acid, 25% of the theoretical. n_D^{25} 1.4455.

Anal.: Calcd for $C_{25}H_{50}O_2$ (382.7) C, 78.47; H, 13.17 %
Found C, 78.29; H, 13.20 %

Table III. Chromatography A.

Chromatography of products obtained on dehydrobromination of ethyl 2DL-bromo-2DL,4D-dimethylheneicosanoate.

Fraction no.	Volume of effluent ml	Weight of fraction mg	Melting point	Optical rotation in light petroleum (micro tube) (l, 1)	
				α_D^{22}	$[\alpha]_D^{22}$
1	10	2			
2	5	2			
3	6	2			
4	6	10	10.5–11°		
5	6	6	8°		
6	6	37	7.2–7.9°	+0.14° (c, 1.9)	+ 7.4°
7	7	120	8.7–9.0°	−0.24° (c, 6.1)	− 3.9°
8	9	108	7–7.5°	−0.74° (c, 4.75)	−15.6°
9	8	71	22.5–23°	−0.64° (c, 3.45)	−18.5°
10	8	45	7°, 22–23°	−0.69° (c, 3.05)	−22.6°
11	15	70			
12	16	57			
13	20	57			
14	40	63			
15	50	56		−0.48° (c, 2.05)	−23°
16	50	27	22–23°[1]		
17	60	14			
18	50	7			
19	50	3			

[1] n_D^{22} 1.4590.

(−)-Ethyl *trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoate (XXIII)

The mixture of ethyl esters (XX) (1.25 g), potassium hydroxide (1 g), water (2 ml), and ethanol (18 ml of 99.5%) was refluxed for 4 hours. After acidification the acids were taken up in ether in the usual manner. The colourless residue obtained after evaporation of the ether was α-brominated by heating with bromine (0.8 ml) and red phosphorus (0.1 g) to 80° for 5 hours. The reaction mixture was cooled to 15°, and ethanol (2 ml) added dropwise. The mixture was kept at 40° for ½ hour, and then at 80° for a further ½ hour. After cooling to room temperature the mixture was extracted with ether. The ether solution was washed with water and dried with sodium sulphate. The ether was distilled off and the residue subjected to dehydrobromination by heating with quinoline (2.5 ml, purified via the acid sulphate and distilled) to 200° (metal bath) for 3 hours. The dark reaction mixture was extracted with ether, and the ether solution washed with dilute hydrochloric acid followed by water. After drying with sodium sulphate the ether was distilled off. The residue was dissolved in light petroleum and passed through a short column containing 15 ml of Al_2O_3 in order to remove the coloured impurities. The colourless solution was then run through a column of 50 ml of aluminium oxide (chromatography A). The results are given in Table III and Fig. 5. All fractions were free from bromine.

Fractions 10–17 contain *(−)-ethyl* trans-*2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoate.* The combined fractions were crystallized from ethanol (10 ml of 99.5%), giving 0.31 g of white crystalline ester of m.p. 23.1–23.8°. After slow solidification (with nucleus) remelting took place at 23.6°–24.0°. As indicated in Table III, the ester is dimor-

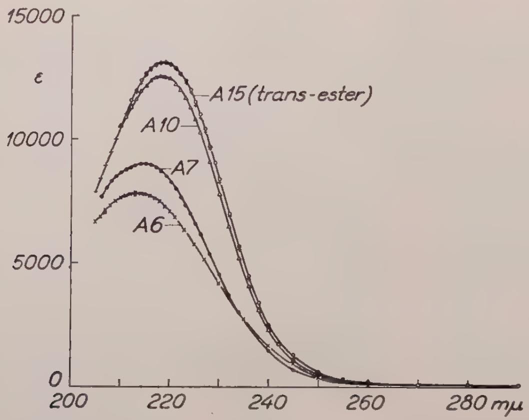

Fig. 5. Ultraviolet absorption of fractions obtained in the chromatography of products obtained on dehydrobromination of ethyl 2DL-bromo-2DL,4D-dimethylheneicosanoate (Table III). For ethyl *trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoate (fraction A15) $c = 5.2 \cdot 10^{-5}$.

phic. The low melting form melts at 7°. n_D^{22} 1.4590. d_4^{22} 0.867. The ultraviolet absorption spectrum is shown in Fig. 5 (A 15); ε_{max} 13100 at 218 mμ. The infrared spectrum is shown in Fig. 2 b. Molecular rotation in light petroleum $[M]_D^{22}$ −84° (see Table III).

Anal.: Calcd for $C_{25}H_{48}O_2$ (380.6) C, 78.88; H, 12.71 %
Found C, 78.32; H, 12.47 %

Table IV. Chromatography B.

Chromatography of material from the early fractions of chromatography A (Table III).

Fraction no.	Volume of effluent ml	Weight of fraction mg	n_D^{22}	Optical rotation in light petroleum (micro tube)	
				α_D^{22} (*l*, 1)	$[\alpha]_D^{22}$
1	10	0			
2	5	0			
3	5	0			
4	5	2			
5	5	25		+0.11° (c, 1.3)	+ 8.5°
6	7	32	1.4544	+0.10° (c, 1.4)	+ 7.1°
7	7	22	1.4543	+0.07° (c, 1.0)	+ 7°
8	7	14	1.4546	+0.04° (c, 0.6)	+ 7°
9	10	18	1.4563	−0.07° (c, 0.8)	− 9°
10	11	15		−0.10° (c, 0.65)	−15°
11	12	14	1.4579		
12	25	20	1.4583		
13	25	10	1.4583		
14	50	7			
15	50	2			
16	50	2			

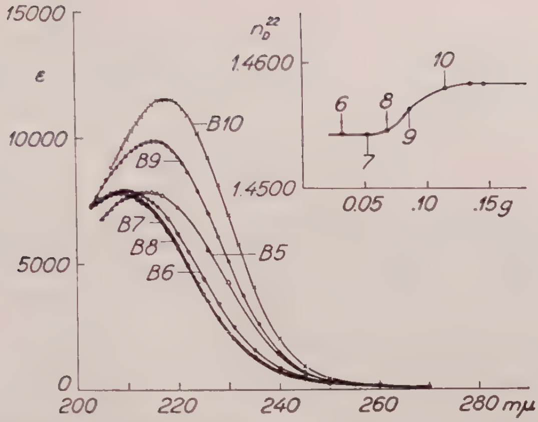

Fig. 6. Ultraviolet absorption and refractive index of fractions obtained in the chromatography (chromatography B, Table IV) of early fractions obtained in chromatography A (Table III and Fig. 5).

Fractions A8 and A9 (0.164 g) were crystallized from ethanol (10 ml), giving 0.092 g of m.p. 21–22.5°, hydrogen number 365, and α_D^{22} −0.80° (*l*,1; *c*, 3.8); $[\alpha]_D^{22}$ −21°. This material evidently consists of slightly impure *trans*-ester. The mother liquor from the crystallization just mentioned was combined with fractions A6 and A7, and the material thus obtained (0.2 g) chromatographed on 20 ml of Al_2O_3. The results are given in Table IV. The ultraviolet absorption spectra of several of the fractions, and the refractive index curve, are shown in Fig. 6. As pointed out in the introduction, it is probable that fractions B7 and B8, with ε_{max} 7.900 at 208 mμ, consist of the *α-methylene ester*. On account of the small amount of material the fractions could not be further investigated. The total yield of pure *trans*-ester was 15% of the theoretical.

(−)-*trans*-2,4D-Dimethyl-$\Delta^{2:3}$-heneicosenoic acid (XXIV)

The ethyl ester (133 mg), potassium hydroxide (0.6 g), water (2 ml), and ethanol (20 ml of 99.5%) were refluxed for 2 hours. The hydrolysis mixture was acidified with hydrochloric acid, extracted with ether and the ether solution washed three times with water. After drying the solution with sodium sulphate the ether was distilled off. The solidified residue was dissolved in 6 ml of light petroleum (b.p. 40°–60°) and crystallization allowed to take place at a temperature of −25°, giving 101.5 mg (82% of the theoretical) of trans-*2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid* (XXIV) in the form of small lustrous plates. The acid melted at 46.0–46.2°. After slow cooling the solidified material remelted at 46.0–46.2°, but after rapid cooling under running water remelting occurred at 49.6–50.2°, indicating the existence of a second crystalline form.

The ultraviolet absorption varies with concentration in a way similar to that of the 2,5D-isomer. ε_{max} 12 600 at 216 mμ ($c = 12.2 \cdot 10^{-5}$ moles per litre) and ε_{max} 11 700 at 215 mμ ($c = 6 \cdot 10^{-5}$). The infrared spectrum is reproduced in Fig. 2 d.

Long crystal spacing (crystallized from light petroleum) 39.3 Å.

Optical rotation:

$\alpha_D^{23} - 0.74°$ (chloroform ; *l*, 1 (micro tube); *c*, 3.38)
$[\alpha]_D^{23} - 21.9°$ $[M]_D^{23} - 77°$

Anal.: Calcd for $C_{23}H_{44}O_2$ (352.6) C, 78.34; H, 12.58 %
Found C, 78.33; H, 12.65 %

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SUMMARY

The synthesis of *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid and the isomeric 2,4D-dimethyl substituted acid is described. In the first mentioned case, (+)-3D-methylnonadecanoic acid, synthesized from (+)-methyl hydrogen β-methylglutarate and palmitic acid via the KOLBE reaction, is converted into (–)-1-iodo-3D-methylnonadecane. Alkylation of ethyl methylmalonate with this iodide gives a mixture of diastereoisomeric 2,5D-dimethylheneicosanoic acids. α-Bromination, followed by dehydrobromination of the methyl ester of the bromo-acid, gives (–)-methyl *trans*-2,5D-dimethyl-$\Delta^{2:3}$-heneicosenoate together with unsaturated isomeric esters. Separation is effected by chromatography on aluminium oxide. The *trans*-α,β-unsaturated ester and the corresponding acid have very low optical rotations.

The synthesis of (–)-*trans*-2,4D-dimethyl-$\Delta^{2:3}$-heneicosenoic acid starts with the preparation of (+)-3D-methyleicosanoic acid from (+)-methyl hydrogen β-methylglutarate and *n*-heptadecanoic acid. A KOLBE synthesis with (+)-3D-methyleicosanoic acid and ethyl hydrogen methylmalonate gives a mixture of stereoisomeric ethyl 2,4D-dimethylheneicosanoates. These are hydrolyzed, and the acids subjected to α-bromination followed by dehydrobromination of the α-bromo ethyl esters. The *trans*-α,β-unsaturated ester is isolated by chromatography on aluminium oxide. This ester and the free *trans*-2,4D-dimethylsubstituted α,β-unsaturated acid have high optical rotations. The molecular rotation of the acid in chloroform solution is $[M]_D^{23} - 77°$.

The physical data recorded include ultraviolet and infrared absorption spectra.

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On the preparation of ethyl *tert*-alkylcyanoacetates

By Arne Brändström and Ingemar Forsblad

The reactions of Grignard reagents with diethyl alkylidenemalonates (1, 2) and ethyl alkylidenecyanoacetates (3–6) have been the subject of many recent investigations. Although most of the investigators state that the yield of the addition product is not changed by a variation of the method given by Birch (3) and independently by Wideqvist (4) and by Alexander, McCollum and Paul (5), it is interesting to note that an older investigation by Hook and Robinson (7), which is not cited by any of the more recent authors except Birch (3), states that cuprous salts increase the yield of the addition product. Since Hook and Robinson's account is rather difficult to find for a person not interested in long-chain compounds, and since the use of cuprous salts as catalysts greatly improves the method for the preparation of ethyl *tert*-alkylcyanoacetates, it was thought that the present investigation of the reaction of Grignard reagents with ethyl *sec*-butylidenecyanoacetate might be of interest.

In the reaction of Grignard reagent with ethyl alkylidenecyanoacetates, the main reactions can be expected to be addition, reduction and condensation. As the reduction may consume one or two moles of the Grignard reagent to one mole of the unsaturated compound, whereas the addition always involves one mole, a variation in the concentration of the Grignard reagent will have a considerable effect on the ratio of the addition product to the reduction product if two moles of the Grignard reagent are involved in the reduction, but little effect if one mole is involved. In order to test this conclusion, the reaction was performed both by adding the cyanoester to the Grignard reagent, in which case the concentration of the Grignard reagent was high, and by adding the Grignard reagent to the ester, in which case the concentration of the Grignard reagent was low. The results are given in Table I and II, from which it is clear that in the case of *n*-propylmagnesium bromide the yields are markedly better when the Grignard reagent is added than in the reverse procedure, but that the results obtained with *n*-butylmagnesium bromide are much less significant. The conclusion drawn is that the addition and the reduction reactions are kinetically very similar but that somewhat better yields can be obtained when the Grignard reagent is added to the unsaturated compound than by the reverse procedure. This result is in conflict with that of Prout (6), who stated that the same yield was obtained in both processes.

The yield of the addition product can be still more improved by adding a little cuprous chloride to the ester before the addition of the Grignard reagent. In this way a yield of 73 per cent of the addition product was secured, compared with 53 per cent without the addition of the cuprous chloride.

Tab. I. The reaction of *n*-propylmagnesium bromide with ethyl *sec*-butylidenecyanoacetate.

Product added	Time for addition min.	Temp.	Addition %	Reduction %
Cyanoester	100	− 5°	27	44
	10	reflux	33	33
Grignard reag.	10	reflux	39	33
	100	− 5°	44	31

Tab. II. The reaction of *n*-butylmagnesium bromide with ethyl *sec*-butylidenecyanoacetate

Product added	Time for addition min.	Temp.	Addition %	Reduction %
Cyanoester	25	reflux	43	25
	150	− 2°	48	32
Grignard reag.	25	reflux	42	22
	180	− 2°	53	27
	75	− 5°	52[1]	27[1]
	30	—2°	73[2]	10[2]
Cyano-ester	30	− 2°	48[3]	25[3]

[1] In this run three times the quantities specified were used.
[2] In this run 0.5 g of cuprous chloride was added to the solution of the cyanoester.
[3] In this run 0.5 g of cuprous chloride was added to the filtered Grignard reagent solution.

That the addition of cuprous chloride is a very important improvement can further be seen from the fact that with *iso*-butylmagnesium bromide the yield of the addition product was 34 per cent using the cuprous chloride catalyst, compared with only 8.5 per cent without it.

It is very important to note that when cuprous chloride (which, being insoluble in ether, must be in the flask) is used as a catalyst the yield is improved only if the Grignard reagent is added to the ester containing the catalyst. In an experiment where the ester was added to the Grignard reagent containing cuprous chloride, the yield was the same as without the catalyst.

In the subsequent preparation of the *tert*-alkylacetic acid from the cyanoester, it was found that although the cyano-group was only hydrolysed with extreme difficulty, the ester group was readily saponified, yielding the cyanoacid, which could then be decarboxylated to form the *tert*-alkylacetonitrile with a good yield in a more convenient way than that given by PROUT (6).

Experimental

Ethyl 2-cyano-3-ethyl-3-methylhexanoate was prepared from the following solutions.

1. 7.3 g of magnesium turnings was covered with 20 ml of anhydrous ether, and a solution of 37 g of *n*-propyl bromide in 100 ml of anhydrous ether added in the usual way to prepare a Grignard reagent.

2.42 g of ethyl 2-cyano-3-methyl-2-pentenoate was dissolved in 50 ml of anhydrous ether.

These two solutions were mixed in the manners indicated in Table I. The reaction mixture was then decomposed by 17 ml of conc. sulphuric acid and an excess of ice. The ether phase was separated and the water layer extracted with 2 × 25 ml of benzene and the combined organic layers washed with 75 ml of a saturated solution of sodium bisulphite, then with 50 ml of water, and finally dried over 2–3 g of anhydrous sodium sulphate. The mixture was then fractionated through a 30 cm Widmer column, the fraction boiling at 102–106°/8 mm collected as the reduction product, and the fraction at 128–132°/8 mm as the addition product. Only a small middle fraction was obtained, usually about 2 per cent of the total amount of the esters.

Ethyl 2-cyano-3-ethyl-3-methylheptanoate was prepared in the same manner as the compound above except that the propyl bromide was replaced by an equivalent amount of *n*-butyl bromide. The reduction product was collected at 103.5–107°/9 mm and the addition product at 136.5–138°/8 mm. The quantities used were twice those mentioned above. The results are given in Table II. In the last two runs in this table 0.5 g of cuprous chloride was added to the liquid in the flask before the addition of the second reagent. The Grignard reagent in this case must be freed from suspended magnesium by filtration.

3-Ethyl-3-methylheptanenitrile

A stirred mixture of 180 g of ethyl 2-cyano-3-ethyl-3-methylheptanoate and 300 ml of 5-N potassium hydroxide was gently boiled in an open beaker for one hour. The clear solution obtained was cooled and acidified with sulphuric acid. The liberated acid was extracted with benzene and dried over anhydrous sodium sulphate. The benzene was removed by distillation and the residue decarboxylated by heating at 200° for 15 minutes. The resulting nitrile was then fractionated under reduced pressure. The yield was 101 g, or 83 per cent of the nitrile boiling at 95–96°/11 mm.

Ethyl 2-cyano-3-ethyl-3,5-dimethylhexanoate

A Grignard reagent was prepared from 17.5 g of magnesium, 96 g of *iso*butyl bromide and 265 ml of anhydrous ether. The Grignard reagent was filtered and added to a well-stirred mixture of 83.5 g of ethyl 2-cyano-3-methyl-2-pentenoate, 1 g of cuprous chloride and 100 ml of anhydrous ether at a temperature of −2° to −3°. The reaction product was treated in the manner described for the *n*-propyl compound. 45.2 g (53.5 per cent yield) of the reduction product b.p. 105–106°/10 mm and 37.5 g (33.5 per cent yield) of the addition product b.p. 136–137°/10 mm were obtained.

3-Ethyl-3,5-dimethylhexanenitrile was prepared by the method given for 3-ethyl-3-methylheptanenitrile from 32.5 g of ethyl 2-cyano-3-ethyl-3,5-dimethylhexanoate and 60 ml of 5-N potassium hydroxide. 16.5 g (75 per cent yield) of a product boiling at 88–89°/8 mm was obtained.

3-Ethyl-3,5-dimethylhexanoic acid was prepared from the nitrile by the method described by PROUT (6). The yield was 73 per cent and the boiling point 134–136°/10 mm.

Anal. Subst. 65.38 mg: 11.76 ml of 0.03230-N NaOH.
17.30 mg: 44.44 mg CO_2, 17.72 mg H_2O.
$C_{10}H_{20}O_2$ (172.26) calc. Equ.wt 172.26; C 69.72 %; H 11.70 %
found Equ.wt 172.10; C 70.20 %; H 11.53 %.

The benzyl-*iso*-thiuronium salt of the acid melted at 127.5–128.5°.

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Die Fällung von Nukleoproteiden, Nukleinsäuren und Nukleotiden durch Dihydrostreptomycin

Von R. Giuliano, M. L. Stein und H. von Euler

Die Hemmung der Chlorophyll-Bildung in keimenden grünen Pflanzen durch Streptomycin ist seit der Entdeckung (Euler[1]) durch eine Reihe von Forschern (Provasoli, Hutner u. Schatz[2]; Schopfer[3]; Quagliariello[4]; Cedrangolo[5]) an verschiedenem Material bestätigt worden. Die Bedeutung des Nachweises der zuerst bei Gerste beobachteten chemisch chlorophylldefekten Pflanzen liegt darin, dass die Kenntnis dieses chemischen Vorganges geeignet ist einen Anhaltspunkt zu geben für das Studium der natürlichen, durch Mendel-Spaltung entstehenden Mutanten.

Die Fähigkeit des Streptomycins, die Chlorophyll-Bildung zu hemmen steht offenbar in Zusammenhang mit der von Euler und Bracco[6] studierten Fällbarkeit des Chloroplastins (A. Stoll) aus Chloroplasten. Ausgehend von diesen Arbeiten haben Euler und Heller[7] systematisch untersucht, welche Nukleoproteide, Nukleinsäuren und Nukleotide durch Streptomycin und durch dessen Spaltprodukte (Guanidinderivate) gefällt werden.

Aus den im Folgenden zu erwähnenden Beobachtungen geht hervor, dass verschiedene Faktoren, besonders die relativen Konzentrationen der Substrate und des Streptomycins die höhere oder geringere Empfindlichkeit der Fällungsreaktion entscheidend beeinflussen.

Hier mag auch gleich die vollständige Hinderung der Streptomycinfällung erwähnt werden, die eintritt, wenn sich gleichzeitig mit dem Streptomycin einige Zeit *Cystein* in Lösung befand, bevor das Substrat zugesetzt wurde. Solche Hemmungen, welche die Bedingung der Umkehrbarkeit erfüllen, sind von besonderem Interesse, da das Cystein zu den Stoffen gehört, welche die biologische Inaktivierung des Streptomycins bewirken[8, 9]. In dieser Gruppe von Stoffen findet man Verbindungen, welche die Sulfhydrylgruppe enthalten, sowie andere, welche sich mit der Aldehydgruppe des Streptomycins verbinden oder die Aldehydgruppe umwandeln[9, 10, 11].

Diese Ergebnisse lassen vermuten, dass im Streptomycin die Aldehydgruppe eine

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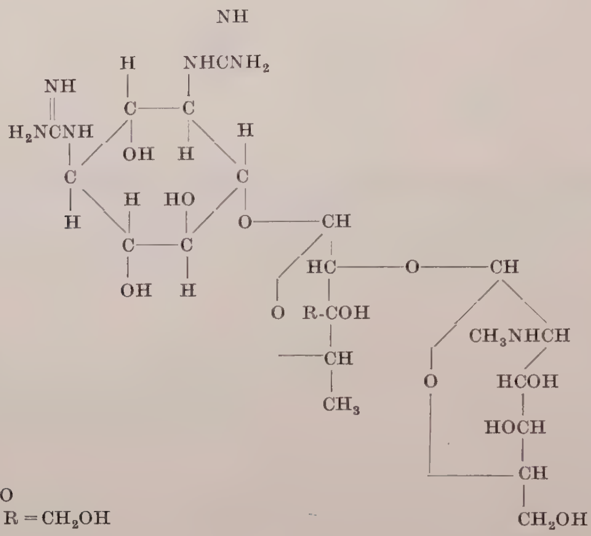

Streptomycin R = CHO
Dihydrostreptomycin R = CH_2OH

wesentliche Rolle spielt. Eine diesbezügliche Aufklärung schien erreichbar, wenn man untersucht, wie sich unter analogen Bedingungen das *Dihydrostreptomycin* verhält, in welchem die Aldehydgruppe durch die Oxymethylgruppe ersetzt ist (siehe obige Formel). Wir haben deswegen die Wirkung des Dihydrostreptomycins auf die Bildung des Chlorophylls in Pflanzen sowie auf die Fällung der Nukleinsäuren, Nukleoproteide und Nukleotide vergleichend untersucht, und zwar mit und ohne Zusatz verschiedener Stoffe.

Per mg Antibiotikum in der Keimlösung ist die Antichlorophyllwirkung des Dihydrostreptomycins schwächer als die des Streptomycins. Im Wesentlichen sind die Antichlorophyll-Effekte der beiden Antibiotika gleichartig; auch beim Keimen von Samen, z. B. von Gerste in Dihydrostreptomycin geeigneter Verdünnung entstehen weisse Keimblätter mit grünen Spitzen.

In Übereinstimmung mit diesen an Dihydrostreptomycin gewonnenen Ergebnissen steht die Tatsache, dass bei dieser Dihydroverbindung die fällende Wirkung auf Nukleinsäuren und deren Derivate ebenfalls weniger ausgesprochen ist und in grossen Verdünnungen, welche bei Streptomycin noch wirksam sind, nur undeutlich zum Ausdruck kommt. Diese verminderte Fällungswirkung macht sich besonders geltend an Nukleoproteiden, welche von Streptomycin noch in einer Verdünnung M/3200 gefällt werden, während Dihydrostreptomycin schon in einer 1/1600 molaren Lösung diese Fähigkeit nicht mehr besitzt.

Die Tabelle 2 zeigt, wie die Fällung bei abnehmenden Konzentrationen des Dihydrostreptomycins umso empfindlicher ist, je kleiner die Konzentration des Substrates ist, eine Erscheinung die noch etwas deutlicher beim Streptomycin hervortritt.

Die Tabelle 3 zeigt den Einfluss von Natriumchlorid auf die Grenzen der Fällbarkeit. Die Konzentrationen des Salzes wurden zwischen 0,01 und 0,2 molar variiert. Das Salz ruft im allgemeinen eine Verkleinerung der fällenden Wirkung in Konzentrationen hervor welche sich bei Dihydrostreptomycin sonst noch wirksam erweisen. Die Unterschiede der Fällungswirkung durch höhere Kochsalzkonzentrationen sind hier geringer.

Tab. 1

Substrate (0,1 %)	Molarität des Dihydrostreptomycins					Kontrolle mit Wasser
	1/200	1/400	1/800	1/1600	1/3200	
Hefen-Ribonucleinsäure .	+ + + +	+ + +	+	–	–	–
Desoxyribonucleinsäure aus Milz	+ + + +	+ + + +	+	–	–	–
Desoxyribonucleinsäure aus Thymus	+ + + +	+ +	±	–	–	–
Lebernucleoproteid A_{1d} .	+ + + +	+ + +	+	–	–	–
Lebernucleoproteid A_{2d} .	+ + + +	+ + +	+	–	–	–

Tab. 2. Einfluss der Konzentration der Ribonukleinsäure

Konzentration der Ribonukleinsäure	Molarirät des Dihydrostreptomycins					Kontrolle mit Wasser
	1/200	1/400	1/800	1/1600	1/3200	
0,2 %	+ + + +	+ + + +	±	–	–	–
0,1 %	+ + + +	+ + +	+	–	–	–
0,025 %	+ + + +	+ + +	+	±	–	–
0,0125 %	+ + + +	+ + +	+	±	–	–

Tab. 3. Hemmung der Fällung der Ribonucleinsäure in 0,1 %-iger Lösung durch Natriumclorid

Molarität NaCl	Molarität des Dihydrostreptomycins					Kontrolle mit Wasser
	1/200	1/400	1/800	1/1600	1/3200	
0	+ + + +	+ + +	+	–	–	–
0,01	+ + + +	+ +	+	–	–	–
0,05	+	+	±	–	–	–
0,1	+	+	±	–	–	–
0,2	+	+	–	–	–	–

Tab. 4. Einfluss des Guanidins und des Harnstoffs auf die Fällung von Ribonukleinsäure in 0,1 %-iger Lösung mit Dihydrostreptomycin

Zusatz	Molarität des Dihydrostreptomycins					Kontrolle mit Wasser
	1/200	1/400	1/800	1/1600	1/3200	
Keine Zusatz	+ + + +	+ + +	+	–	–	–
Guanidin (0,01 M)	+ + +	+ +	±	–	–	–
Harnstoff (0,01 M) . . .	+ + + +	+ + +	+ +	±	–	–

Tab. 5. Einfluss von Cystein-Zusätzen auf die Fällbarkeit der Ribonukleinsäure durch Dihydrostreptomycin

Zusatz	Molarität des Dihydrostreptomycins					Kontrolle
	1/100	1/200	1/400	1/800	1/1600	
NaCl (0,01 M)	+ + + +	+ + + +	+ +	+	–	–
Cystein (0,01 M)	+ + +	+ +	+	+	–	–
Cystein (0,01 M) nach 24 Stunden	+ + +	+ +	+	+	–	–

Der Zusatz von Guanidin welcher bereits in der früheren Arbeiten über Streptomycin besprochen wurde, ist veranlasst durch die Gegenwart von Guanidingruppen in diesen antibiotischen Stoffen, durch welche diese nach der Theorie von Woods-Fildes als biologischer Verdrängungsstoff der betreffenden Antibiotika wirken könnte.

Guanidin bedingt nur eine mässige Verminderung der Fällungsgrenzen (Tabelle 4). Umgekehrt ruft der Zusatz von Harnstoff in Analogie mit dem Befund von Euler und Heller am Streptomycin einen die Fällung von Nukleinsäuren begünstigenden Effekt hervor. Tatsächlich begrenzt Harnstoff die Fällung bei grössere Verdünnung des Dihydrostreptomycins.

Ein noch bemerkenswerterer Unterschied zwischen den beiden antibiotischen Stoffen macht in ihrem Verhalten gegen Cystein geltend. Schon oben haben wir darauf hingewiesen, dass das Cystein die antituberculotische Wirkung des Streptomycins hemmt. Diesem Verhalten entspricht das Fehlen der Fällungswirkung gegenüber Nukleinsäuren, wenn sich diese in der gleichen Lösung wie das Cystein befinden. Die Untersuchungen von Ingrao und Vella[1] über die antituberkulotische Wirkung des Dihydrostreptomycins haben gezeigt, dass Cystein *diese* Wirkung nicht hemmt.

Die Versuche über die Fällung des Dihydrostreptomycins in Gegenwart von Cystein haben bewiesen — wie aus der Tabelle 5 hervorgeht — dass Cystein einen recht geringen Einfluss auf die Fällungsreaktion ausübt. Dieses Verhalten ändert sich nicht, wenn die Lösungen des Dihydrostreptomycins und des Cysteins 24 Stunden vor dem Versuch gemischt und bei 4° gehalten werden. Unter letzterer Bedingung hört beim Streptomycin die Fähigkeit zur Fällung auf.

Unter anderen Verbindungen, welche eine SH-Gruppe enthalten, haben wir die *Thioglykolsäure* auf ihr Verhalten gegen Streptomycin und Dihydrostreptomycin untersucht. Auf ersteres beobachtet man eine Wirkung nur dann, wenn die Lösungen vorher 24 Stunden gemischt waren. Dieser Effekt zeigt sich viel weniger ausgeprägt als der von Cystein ausgeübte (Tab. 6). Auf Dihydrostreptomycin hat thioglykolsäures Na überhaupt keinen Einfluss.

Dieser Unterschied macht es sehr wahrscheinlich, dass die Zwischenwirkung zwischen Cystein und Streptomycin von einer durch die Aldehydgruppe bedingten Komponente abhängt. Dabei wird abgesehen von anderen Ursachen chemischer und biologischer Art, welche von Chester J. Cavalito[2] erwähnt und besprochen wurden.

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Tab. 6. Einfluss von Zusätzen von Na-Thioglykolat auf die Fällbarkeit der 0,1 % Ribonukleinsäure durch Streptomycin (pH 7,4)

Zusatz	Molarität des Streptomycins					Kontrolle
	1/100	1/200	1/400	1/800	1/1600	
Keine Zusatz	+ + + +	+ + + +	+ + + +	+ +	+	–
Na-Thioglikolat (0,01 M) nach 24 Stund.	+ + + +	+ + + +	+ + +	+	–	–
Cystein (0,01 M) nach 24 Stund.	–	–	–	–	–	–

Methodik

Unsere Versuche wurden ausgeführt mit einem Dihydrostreptomycin-Preparat der Firma Squibb, an welchem auch die analytischen Kontrollen ausgeführt wurden. Die wässrige Lösung wurde durch Natronlauge auf die Azidität pH 7,4–7,6 gebracht. Die Substrate entsprechen denen von Euler und Mitarb.[1] verwendeten.

Die *Nukleoproteide* der Leber wurden erhalten aus den Extrakten der von Zellkernen und Mitochondrien durch Zentrifugierung befreiten Leber und sie wurden nach Dialyse beim isoelektrischen Punkt gefällt. Das Präparat A_{1d} war einmal, das Präparat A_{2d} zweimal umgefällt.

Als *Nukleinsäuren* wurden Präparate des Institutes verwendet, welche teils nach Brachet[2], teils nach Bredereck und Caro[3] aus Milz dargestellt worden waren. Die Lösungen der Substrate wurden unter Zusatz von Natronlauge dargestellt und auf die Azidität pH 7,4 gebracht. Nach Zusatz der Dihydrostreptomycin-Lösungen zu der Lösungen der Substrate und anderer untersuchter Substanzen wurde stets das pH geprüft und auf den oben erwähnten Normalwert gebracht.

Bei den Versuchen mit Cystein wurde ein Vergleich mit einer M/100 NaCl-Lösung ausgeführt um festzustellen ob die verwendete M/100 NaCl-Lösung mit der Azidität des mit Natronlauge neutralisierten Cysteins übereinstimmt.

Die Trübungsmessungen wurden ausgeführt mit einem Turbidimeter in dem als Nullpunkt eine Lösung verwendet wurde, hergestellt aus dem verdünnten Substrat. Die Intensität des durchscheinenden Lichtes ist ausgedrückt durch die von Heller und Euler angenommenen Symbole, welche auf einer graphischen Interpolation beruhen.

ZUSAMMENFASSUNG

Die hemmende Wirkung des Dihydrostreptomycins auf die Bildung des Chlorophylls in keimenden Samen tritt mit Sicherheit ein, aber in einem Grad, welcher geringer ist als der an Streptomycin beobachtete.

Die fällende Wirkung des Dihydrostreptomycins auf Nukleoproteide und Nukleinsäure ist im Vergleich zu der des Streptomycins geringer.

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Der Zusatz von Cystein hindert nicht die Fällung der genannten Substrate durch Dihydrostreptomycin, zum Unterschied von Streptomycin. Diese Tatsache kann mit dem Befund von INGRAO und VELLA in Zusammenhang gebracht werden, dass das Cystein die antituberkulotische Reaktion des Dihydrostreptomycins nicht hemmt, wohl aber die des Streptomycins.

Der Zusatz von Natriumchlorid, Guanidin und Harnstoff zeigt keine deutlichen Unterschiede in den Lösungen der beiden Antibiotika.

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